NATIONAL INSTITUTES OF HEALTH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
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Under its congressional mandate, the National Heart, Lung, and Blood Institute

--conducts and supports fundamental research on the heart, blood vessels, blood, and lungs and on the diseases that afflict them;

--develops and evaluates new or improved means for the prevention, detection, diagnostic evaluation, and treatment of these diseases;

--encourages widespread application of proven new techniques by the research and medical communities;

--provides support for training research workers, clinicians, and teachers in the cardiovascular, blood, and pulmonary fields; and

--informs the general public and health professionals about research and clinical advances arising from Institute programs.

Some highlights of progress during the year in major NHLBI program areas are briefly described in the sections that follow.

Heart and Blood Vessel Diseases

Major heart and blood vessel diseases addressed by the Institute programs include arteriosclerosis, coronary heart disease, hypertension, cerebral vascular disease, and congenital and rheumatic heart diseases. Other research conducted and supported by NHLBI deals with arrhythmias, cardiomyopathies, heart failure and shock, peripheral vascular diseases, medical devices and technology, epidemiology and biometry, and behavioral medicine.

An important facet of this research is identification of factors that increase susceptibility to heart and blood vessel diseases as well as evaluation and application of countermeasures against modifiable risk factors, the goal being to avert or delay the onset of disabling or potentially lethal manifestations of these disorders.

Elevated blood levels of low-density lipoproteins (LDL), the principal carriers of cholesterol in the blood, are associated with increased risk of premature arteriosclerosis, whereas a robust high-density lipoprotein (HDL) fraction is thought to confer some measure of protection against the disease.

33
98
552
980

Investigators in the Framingham Offspring Study compared LDL and HDL cholesterol values among 1,312 males aged 35 to 54. Of these, 43 had clinical coronary heart disease, the remainder did not. When LDL or HDL cholesterol were considered separately, neither provided any clear-cut distinction between subjects with and those without CHD, but considering them together, the investigators noted that:

--the presumably protective combination of low LDL cholesterol and high HDL cholesterol was more than twice as common among subjects without CHD as among subjects with CHD (52 percent versus 21 percent, respectively).

--the presumably atherogenic combination of high LDL cholesterol and low HDL cholesterol was three times as common among subjects with CHD as among those without CHD (21 percent versus 7 percent, respectively).

Regular physical exercise affords one means of raising HDL levels, though there is some evidence that it works better for men than for women. Among men, a recent study indicated, the amount of habitual exercise appeared to be directly related to the HDL levels observed.

The investigators compared HDL levels in marathon runners, joggers, and inactive men. The marathon runners were training strenuously and averaged running some 40 miles per week; the joggers averaged about 11 miles a week; and the inactive men neither jogged nor ran on a regular basis.

The marathon runners had the highest HDL levels (64.76 ± 14.25 mg/dl), the sedentary individuals the lowest (43.31 ± 14.24), and the joggers' levels were in between (58.04 ± 17.70), though nearer those of the marathon runners. The most active men also had the lowest blood levels of cholesterol and triglycerides, and the least active had the highest levels of the lipids. Though there were some dietary differences among the three groups, they did not appear to account for the observed differences in HDL levels.

More modest exertions can also improve the HDL picture. Other investigators compared HDL levels among heart-attack survivors who participated in an exercise rehabilitation program, heart-attack survivors who did not participate, and normal controls. Initially, both groups of MI patients had HDL levels (35.2 ± 7.9 mg/dL) significantly lower than those of the controls. With training (45 minutes of walking, jogging, and calisthenics three times a week), HDL levels among program participants rose to 40.0 ± 8.4, a level significantly higher than that of nonparticipant and similar to that of the controls. The higher HDL levels in the rehabilitation group were achieved after only 1 week of participation in the training program and persisted throughout the 6 months of the study.

Examination of the blood vessels of the retina is routine procedure in the evaluation of patients with hypertension or diabetes. However, a recent study suggests that, in the absence of either disease, changes in the retinal vessels may be independently associated with atherosclerosis and may provide a sensitive indicator of the presence and severity of coronary heart disease.

Seventy patients, neither hypertensive nor diabetic but who were scheduled for coronary angiography because of chest pain, were initially examined by an ophthalmologist. He checked the retinal vessels for arteriolar light reflex changes, decreased blood vessel caliber or increased tortuosity, and for arteriovenous crossing defects.

Of 47 patients subsequently found to have CHD on angiography, 46 had exhibited abnormal light reflexes on on funduscopic examination. Of 23 patients without CHD, light reflexes were normal in 14 and the other 11 exhibited only slight changes (grade 2 or less). In general the grade of the light reflex changes correlated with the extent of the coronary disease.

Abnormal light reflexes proved to be the most sensitive indicator; the other abnormalities, though occurring much more frequently in CHD patients than in the normal subjects, ranged in sensitivity from 19 percent for blood vessel tortuosity to 68 percent for crossing defects. Only 1 patient with CHD exhibited no retinal abnormalities at all, versus 13 of those without CHD.

The percentage of women in the U.S. labor force increased from 23 percent in 1950 to 42 percent in 1978, with most of this increase accounted for by working wives. Some have surmised that working women must often contend with higher levels of stress than housewives usually experience and they have been apprehensive that this might increase the working women's vulnerability to coronary heart disease.

Recent results from the Framingham Heart Study indicate that the working women in the Framingham population were under more stress than were the housewives but, overall, did not have significantly higher incidence rates of CHD (7.8 percent versus 5.4 percent, respectively). However, certain subgroups of working women did appear to be at appreciably higher risk: among clerical workers, the incidence of CHD (10.6 percent) was nearly twice that of housewives and, among clerical workers with children, it was nearly three times as high (15.4 percent). At highest risk were clerical workers with children and who were married to blue collar workers (CHD incidence: 21.3 percent).

Factors associated with higher CHD incidence rates included non-supportive bosses, suppressed hostility, dead-end jobs or restricted job mobility, and three or more children--the last suggesting that the combined roles of wife, mother, and breadwinner may be especially taxing.

Another factor may be the Type A behavior pattern, characterized by aggressiveness, immersion in deadlines, and a chronic sense of time urgency. Working women were much more likely to report Type A behavior than were housewives; and, in another study among Framingham subjects, Type A women developed twice as much CHD and three times as much angina as did their more placid Type B sisters.

In 1959 the Build and Blood Pressure Study, based on the actuarial experience of U.S. insurance companies over the period 1935-1953, indicated a direct relationship between mortality and body weight. In general, the data indicated, the heavier the individual in relation to height, the greater his risk of death, whereas this risk was lowest among those well below average weight. The report provided the basis for tables of desirable weights prepared by the Metropolitan Life Insurance Company and widely used by physicians.

Recently, a study among individuals enrolled in the Framingham Heart Study arrived at somewhat different conclusions. The results indicated that mortality rates were lowest in the average weight range and were higher at either extreme of the weight-height spectrum: that is, among individuals substantially above or below average weight.

The Framingham findings with respect to obesity were not surprising in view of the atherogenic traits commonly associated with overweight: higher blood presure; a higher prevalence of diabetes; and higher blood levels of lipids, glucose, and uric acid.

However, the higher mortality noted among the leanest subjects is more puzzling. Although a higher proportion of individuals in this group were smokers, the excess risk associated with the habit did not fully explain their higher mortality, nor did weight loss possibly due to some debilitating or potentially life-threatening illness.

The study results do not contradict the plethora of evidence that sensible programs of weight reduction are probably beneficial in obese subjects, but they do raise questions about the value of such regimens in persons already at or near average weight.

The registry compiled in conjuction with the Coronary Artery Surgery Study (described later) provided a group of 8,807 individuals from whom extensive data on risk factors had been collected and in whom subsequent coronary angiograms provided objective evidence of the presence or absence of clinically significant coronary heart disease.

Accordingly, the investigators sought to determine which individual factors and combinations of factors were most strongly correlated with the findings at arteriography. Their analysis showed that age, male gender, cigarette smoking experience, and blood cholesterol levels were the best overall predictors, though a family history of CHD, hypertension, and diabetes had significant predictive value in some subgroups of patients.

Increasing age and male gender were very powerful risk factors, eclipsing any others among the oldest group of male subjects.

The risk associated with smoking and elevated blood cholesterol was most pronounced among younger subjects. Among subjects under 45 who proved to have coronary heart disease upon arteriography, 9 of every 10 were either smokers or ex-smokers. Among women under 55 and males under 35, the combination of elevated cholesterol and smoking increased CHD risk more than fourfold. Conversely, CHD was rarely found among women under 45 with neither of these risk factors.

Though the study demonstrated significant relationships between certain risk factors and angiographically documented coronary heart disease, the investigators feel that risk factor profiles have only limited value for forecasting the presence or extent of the disease in individual patients.

On two occasions during the past year, cancer came up for discussion in the context of NHLBI program activities. The first arose in conjunction with NCI test results indicating that reserpine demonstrated carcinogenicity in mice.

One of the earliest of "modern" blood pressure drugs, reserpine has been in clinical use for more than 30 years. Today, despite a wide variety of agents that have come along since, reserpine remains a favored drug in the treatment of mild to moderate hypertension.

Earlier, reports from several clinical studies had suggested a possible association between reserpine use and increased occurrence of breast cancer in women, but a number of other studies found no such association. Very recently, investigators from the Mayo Clinic examined the relationship, if any, between antihypertensive therapy, especially reserpine and thiazides, and breast cancer among 2,000 hypertensive women in the Rochester, Minnesota area.

Using extensive data on the incidence of breast cancer in the Rochester area gathered during an earlier epidemiological survey plus similar data compiled by the Connecticut Tumor Registry, the investigators compared expected to observed cases of breast cancer in the study population both before and after exposure to reserpine and/or thiazides. The comparison disclosed no evidence of any association between reserpine use and breast cancer. In fact, the ratio of observed to expected cases was lowest among reserpine-treated women, notably among those with longer-term exposure to the drug.

The results of this and other studies suggest that if reserpine is, in fact, carcinogenic, its cancer-causing potential in humans must be very low indeed. In terms of relative risk, any threat of cancer from reserpine use is probably miniscule compared with the threats posed by uncontrolled hypertension, though, if need be, other agents can readily be substituted for reserpine in the antihypertensive regimen. FDA has reviewed evidence for and against any reserpine-cancer connection and has not withdrawn the drug from prescription use.

In February 1980 the Institute convened a panel to review mortality from noncardiovascular causes in eight NHLBI-supported epidemiological studies. Of special concern were data from Framingham, the Honolulu Heart Study, and the Puerto Rico Heart Health Study suggesting that very low serum cholesterol levels may be associated with some increase in risk from various types of cancer.

The Framingham data indicated that males with cholesterol levels below 190 mg/dl had an incidence of colon cancer three times that of males with higher levels, the incidence of this and other cancers decreasing with rising cholesterol levels. The Honolulu Study noted excess deaths from stomach, colon, liver, and lung cancer among males with cholesterol levels below 180, and the Puerto Rican study noted excess cancer deaths among rural males and urban males aged 45-54 with levels below 195, these rates being highest among those with levels below 165. Other studies reviewed by the panel disclosed no such associations.

The panel concluded the available data were too limited and too inconsistent to substantiate any causal relationship between low cholesterol levels and increased cancer risk, adding that the apparent relationship observed in several studies may, in fact, be reflecting effects on blood lipids of inapparent or silent malignancies. However, they recommended continued investigation of the matter, including re-examination of data from other population studies to see whether similar or dissimilar cholesterolcancer relationships might surface. The additional data will be assessed at a second meeting of the panel next spring.

Various epidemiological studies have indicated that bruits in the carotid arteries traversing the neck are an important risk factor for stroke. Because of this association, some vascular surgeons have opined that prolonged, high pitched mid-carotid bruits warrant arteriographic evaluation even if the patient has experienced no symptoms, with endarterectomy recommended if significant narrowing is found in the internal carotid artery.

Data from the Framingham Heart Study indicate that carotid bruits in asymptomatic subjects were, in fact, associated with a stroke rate more than twice that expected on the basis of age and sex. However, more often than not, strokes occurring in these subjects could not be ascribed to carotid artery disease because they either involved a different vascular territory or, in many instances, resulted from ruptured aneurysms or migrant clots from the heart or elsewhere.

Carotid bruits were also associated with a twofold increase in the incidence of heat attacks and higher general mortality, with most deaths due to cardiovascular causes. Those observations led the investigators to conclude that carotid bruits are more often a general, nonfocal sign of advanced arteriosclerosis than a harbinger of local arterial stenosis preceding cerebral infarction.

In another study, involving 6-year followup of subjects over 45 with carotid bruits, but who had not experienced previous strokes, transient ischemic attacks or overt symptoms of coronary heart disease, carotid bruits were associated with a high stroke risk for men but not for women. As in the Framingham Study, the correlation was poor between the location of the bruit and the type of stroke that subsequently occurred, and bruits were also found to increase risk of death from coronary heart disease in men.

The investigators concluded that asymptomatic bruits are indicators of systemic vascular disease but, of themselves, do not justify invasive diagnostic procedures or aggressive surgical approaches.

The Hypertension Detection and Followup Program, completed last year, clearly demonstrated the health benefits of good blood-pressure control in hypertension, whatever the subject's initial blood-pressure level, age, sex, or color. The study involved some 11,000 participants, selected from among nearly 160,000 people initially screened in 14 communities. The study population, aged 30-69, contained nearly equal numbers of men and women, blacks and whites, and represented all strata of high blood pressure from borderline to severe.

Half the subjects received an aggressive, highly systemized program of therapy administered by HDFP clinics. Special efforts were made to secure the patients' full cooperation and faithful adherence to prescribed treatment. The control group was referred to their usual sources of medical care; however, these subjects were seen annually at HDFP clinics and most were under drug treatment throughout the 5-year followup period.

Even so, after 5 years, the clinic patients had a 17 percent lower mortality rate from all causes than did the control group and an 18 percent lower mortality rate from cardiovascular disease. Especially gratifying were the results achieved by the clinics among patients with borderline to mild hypertension (diastolic pressures of 90-104). Clinic patients had a 20 percent lower mortality rate from all causes than did controls in the same blood pressure stratum and a 26 percent lower morality rate from cardiovascular disease.

Whether to treat borderline or mild hypertension had been a controversial question in the medical community, but the HDFP results demonstrated unequivocally that effective treatment of mild hypertension can prolong life and that the health benefits clearly outweigh any attendant risk of side effects in drug-treated patients.

Other clinical trials involving interventions against modifiable risk factors are still in progress.

The Coronary Primary Prevention Trial involves 4,000 men with Type II hyperlipoproteinemia (characterized by elevated blood cholesterol) but with no clinical evidence of coronary heart disease at entry into the study. It is evaluating a cholesterol-lowering diet or the same diet supplemented with cholestyramine (a cholesterol-lowering drug) in reducing expected rates of morbidity or mortality from coronary heart disease in this highrisk group.

The Multiple Risk Factor Intervention Trial involves nearly 13,000 men at heightened risk from coronary heart disease because of various combinations of elevated blood cholesterol, elevated blood pressure, and/or cigarette smoking. Any one of these factors roughly doubles CHD risk, and the presence of all three may run it up by a factor of ten or more. We are hopeful that systematic modification of these factors over a 6-year period will produce substantial reductions in that risk and will be reflected in lowered CHD mortality rates.

Still other NHLBI-supported trials are concerned with secondary prevention: that is, evaluation of measures to reduce disability and improve long-term survival among persons already victimized by atherosclerosis or its complications.

The recently completed Aspirin-Myocardial Infarction Study sought to determine whether daily doses of aspirin would reduce the threat of recurrent heart attacks and the number of cardiac deaths among 4,200 patients who had previously sustained one or more such attacks. Because a high proportion of heart attacks are thought to be precipitated by clotting phenomena in the coronary arteries and because aspirin inhibits the aggregation of blood platelets—thought to be an important early step in clot formation—it was hoped that aspirin might confer significant protection against such clotting complications and so improve long-term survival in the treatment group as compared with patients receiving a placebo.

The study results indicated otherwise: the aspirin-treated group fared no better than the placebo group. Actually, total mortality and heartattack deaths were slightly higher among aspirin-treated patients; and, though they had somewhat fewer nonfatal recurrent attacks and suffered fewer strokes and transient ischemic attacks than did the placebo group, they also had a substantially higher incidence of gastric symptoms. Conclusion: based on the study results, aspirin could not be recommended as a routine prophylactic measure for heart-attack survivors.

Another study on antiplatelet drugs in secondary prevention yielded slightly more encouraging results. The study compared aspirin or aspirin plus dipyridamole with a placebo in 2,026 patients who had previously sustained heart attacks. Though differences between drug-treated and placebo groups did not quite reach statistical significance, both the aspirin and aspirin plus dipyridamole groups had somewhat lower rates for total mortality, CHD mortality, and nonfatal coronary events than did the placebo group. Further data analysis suggested that the benefits of either drug regimen were greatest for patients whose therapy was begun within 6 months of the attack and that any benefits accruing to antiplatelet therapy appeared to be limited to the first 2 years of treatment.

The Beta-Blocker Heart Attack Trial is evaluating another type of drug that may provide long-term protection to heart attack survivors. Propranolol, the drug being tested, has a number of potentially beneficial effects: it reduces heart rate and blood pressure, reduces the cardiac workload, is a blood vessel dilator, and protects against certain types of arrhythmias. The study, initiated in 1977, is currently in the recruitment and intervention phase.

The Multicenter Investigation for the Limitation of Infarct Size (MILIS) is investigating the effectiveness of propranolol or the enzyme hyaluronidase in salvaging threatened, but still viable heart muscle when infused early after the onset of acute heart attacks. It is hoped that either or both agents will reduce the amount of heart muscle irreversibly damaged and thus decrease disability and improve after the heart attack Entry of patients into the study began August 1978; over 1,000 will eventually participate.

The Coronary Artery Surgery Study is comparing medical management of advanced coronary heart disease with coronary bypass surgery, a procedure that has come into widespread use during recent years for the treatment of intractable or unstable angina pectoris. Among still controversial questions that this study will attempt to answer: 1) Under what circumstances does the procedure improve the patient's life expectancy? 2) Under what circumstances does coronary artery bypass reduce the heart-attack risk of patients with advanced coronary obstructions or the risk of recurrent attacks in patients who have previously experienced them? 3) What is the quality of life over an extended period for patient undergoing the procedure as compared to those receiving only medical therapy? Recruitment of 780 patients ended in June 1979; in addition 24,188 patients were enrolled in the CASS Registry. All patients will be followed for 5 years. Two reports were released in the past year from registry studies on the complications of coronary arteriography and on the results of exercise stress testing.

Angina pectoris affects about two million Americans. In most of these subjects, the underlying cause is severe narrowing of one or more coronary branches by arteriosclerosis. However, recent evidence indicates that coronary artery spasm may play a larger role in angina attacks than had previously been supposed.

Variant angina, also called Prinzmetal's angina, is thought to affect about 5 percent of angina victims. This condition can produce episodes of severe chest pain in subjects with little or no coronary arteriosclerosis. Here coronary spasm is clearly the precipitating factor. But arteriosclerotic arteries may also undergo spasm. Indeed, they may be more susceptible to spasm than apparently normal ones. Thus many cases of angina attributed to coronary arteriosclerosis may involve coronary spasm as an important component--perhaps as many as 25 to 30 percent of all cases.

Previously, coronary spasm had been difficult to demonstrate. However, when spasm is suspected as a cause of angina, the cold pressor test or intravenous or intracoronary infusions of ergonovine maleate (a blood vessel constrictor) will usually provoke spasms that reproduce the patient's symptoms and cause characteristic ECG changes. The latter test is not without hazard and so should be done only in a catheterization lab, ICU, or similar facility where serious arrhythmias or other potential complications of induced spasm can be dealt with promptly.

The causes of coronary spasm are unknown; but one suspect in spasm of arteriosclerotic arteries is platelet aggregation at sites of arteriosclerotic lesions, followed by platelet release of thromboxane A_2, a potent blood-vessel constrictor. Conceivably, aspirin, an inhibitor of thromboxane A_2 production by platelets, may be useful in the prevention of spasm due to this cause, though this remains to be demonstrated.

Also suspected as a cause of coronary spasm are localized aberrations of calcium metabolism that increases the influx of this ion into muscle fibers of the arterial wall. This may stimulate the contractile machinery of these fibers to an excessive degree, producing hypertonicity and spasm.

Nitrates or beta adrenergic blocking agents, such as propranolol, though highly effective against typical angina, have enjoyed less success against angina due to spasm. However a promising new group of drugs, collectively called calcium antagonists, are currently undergoing clinical evaluation. One of these agents, nifedipine, appears to be especially effective against angina due wholly or in large measure to coronary spasm.

In a study among 127 patients with documented coronary spasm and whose angina had responded poorly to conventional antianginal drugs, nifedipine reduced the mean number of anginal episodes from 16 per week to 2 per week. Of these patients, 63 percent experienced complete relief from angina while on nifedipine therapy and only 17 patients failed to show a 50 percent or greater reduction in the frequency of attacks. Ten of the latter were receiving lower doses of the drug or were taking it less frequently than most of the study subjects. Side effects of nifedipine included hypotension, dizziness, flushing, and edema of the feet and ankles. These were severe enough to force discontinuation of the drug in 20 of the patients.

Calcium antagonists are thought to work by slightly impeding the movement of calcium into arterial and heart muscle cells. In the arteries this results in some decrease in blood vessel tone and inhibition of spastic contraction. In the heart this is thought to decrease heart-muscle contractility and thus reduce the heart's oxygen requirements.

In addition to providing more effective means of treatment for variant angina, some of the calcium antagonists show promise as antiarrhythmic agents. They may also provide still another approach to limiting or reducing the amount of irreversible heart-muscle damage resulting from acute heart attacks.

A technique called percutaneous transluminal coronary angioplasty (PTCA) may provide an alternative to coronary artery bypass surgery in some 5 to 10 percent of patients now considered surgical candidates. It is most likely to be effective in single vessel disease (except the left main coronary artery).

Ideally the obstruction should be well localized and of relatively recent origin, so that the atherosclerotic deposit has not become fibrous or calcified. In such cases, a balloon tipped catheter may be passed through the site of obstruction under fluoroscopic guidance and the sausage-shaped balloon inflated briefly, compressing the malleable lesion against the blood vessel wall and thereby increasing the size of the blood channel.

Though clinical experiences with PTCA is still limited and its long-term benefits remain to be assessed, the number of U.S. centers employing the technique in carefully selected patients is on the increase. Currently, dilation is successful about 55 percent of the time. PTCA poses some risk: complications have included coronary artery dissection, heart attacks, and, in some instances, death. Emergency coronary bypass surgery has occasionally been necessary when PTCA was unsuccessful. However, the technique has appeared sufficiently promising for the Institute to encourage further laboratory and clinical research on PTCA. NHLBI has also established a registry to facilitate further evaluation of the effectiveness of the procedure and to define its limitations.

Other applications of transluminal angioplasty have been reported. One team employed the procedure to treat ischemia of the extremities resulting from stenoses in the iliac, femoral, or popliteal arteries of 27 patients. Dilation was successful in 30 vessels in 23 patients, all of whom experienced relief of symptoms. During followup periods of 3 to 10 months, stenosis recurred in two iliac arteries (necessitating surgery) and in one femoral artery, which was successfully redilated.

Another team employed transluminal angioplasty in 10 patients to restore normal bloodflow in autogenous vein grafts previously inserted to bypass obstructions in arteries of the lower limbs, but which had themselves developed stenoses 6 to 28 months after surgery. Eleven of 12 lesions were dilated successfully, with restoration of strong pedal pulses in 8 patients and improvement in 2 others. In one patient, only partial dilation could be achieved and he subsequently required reoperation. In another, some restenosis occurred 2 months after dilation, though the graft remained open. In the other patients, all grafts remained patent and the patients had remained symptom free over followup periods of 1 to 7 months.

Secondary hypertension often stems from obstructions in the renal arteries supplying blood to the kidneys. Such obstructions may produce elevated blood pressure by excessive stimulation of the renin-angiotensin-aldosterone system. Often, surgical relief of partial arterial obstructions of this sort has restored normal kidney function and also corrected the associated hypertension. Previously, however, if renal artery obstruction appeared to be total and the affected kidney nonfunctional, the usual approach was to remove the affected kidney.

However, results from a recently reported study indicate that even seemingly hopeless kidney dysfunction (as gauged by conventional renal function tests) may be correctible by restoration of normal renal bloodflow. It appears that, if the renal artery occlusion develops gradually, a collateral circulation to the blood-deprived kidney may develop from such vessels as the periureteral, peripelvic, and/or adrenal arteries. This may be sufficient to keep the kidney viable even when it remains nonfunctional.

Employing endarterectomy or various bypass procedures, the investigators restored renal bloodflow to kidneys rendered nonfunctional by renal artery obstructions in 15 patients. In 10, the viability of the ischemic kidneys was established by biopsies obtained at operation. Renal bloodflow was restored in all patients, with recovery of good renal function in 13. At operation, all patients had renovascular hypertension, which was cured in 10 patients and improved in the other 5, including the 2 whose kidney function was not restored by the operation.

Direct current cardioversion plus the availability of an increasing variety of antiarrhythmic drugs have made the termination of most cardiac arrhythmias a relatively simple matter, but preventing their recurrence may pose greater difficulties. Continuous antiarrhythmic drug protection is often complicated by adverse side effects or problems of patient compliance, whereas repeated hospitalizations for intensive drug therapy or cardioversion are both expensive and inconvenient.

NHLBI grantees report that intermittent use of an antiarrhythic drug "cocktail," which the patient takes only at onset of the arrhythmia, is often effective in controlling recurrent episodes of atrial fibrillation or flutter and ventricular tachycardia.

The drug "cocktail" was individualized for each patient during hospitalization for an arrhythmia episode. For atrial arrhythmias it usually contained digoxin, quinidine, disopyramide, propranolol, or various combinations of these agents. For ventricular arrhythmias it contained quinidine, quinidine plus propranolol, or procainamide. Each cocktail also included a mild sedative. The patient's response to the selected medications and dosages was carefully evaluated while he was still in the hospital, and before discharge he was also instructed in carotid sinus massage and Valsalva maneuver, which sometimes terminate such arrhythmias without medication.

The investigators judged the new approach effective in 24 of 32 patients. Even in those who subsequently proved to need continuous drug protection because of frequent recurrences, the cocktail was helpful in the treatment of breakthrough episodes. The investigators conclude that such intermittent drug therapy offers a safe, effective, and less costly approach to the management of episodic arrhythmias.

Ventricular aneurysms, which may develop in the wake of acute heart attacks, are sometimes a source of recurrent and dangerous arrhythmias—chiefly ventricular tachycardias—that are often resistant to drug therapy.

Surgical excision of the aneurysms sometimes cures these arrhythmias or reduces requirements for antiarrhythmic drugs; but, because the spot of electrically irritable tissue responsible for the arrhythmia may lie adjacent to the aneurysm rather than in it, such surgery is successful only about half the time. More extensive resection may catch the irritable spot, but at the expense of functional heart muscle.

NHLBI grantees sought to identify and exise these irritable sites in 31 surgical patients. They recorded electrocardiograms at 50 to 90 spots on the heart surface after inducing rapid ventricular rates by programmed electrical stimulation. The spot of earliest depolarization indicated the irritable site, which was excised completely in 27 patients but could be only partially excised in three others. The aneurysms were also excised in 27 patients and coronary bypass grafts placed in 21. There were two operative deaths and three late deaths.

Of the 25 survivors followed for periods up to 28 months, none has experienced recurrent arrhythmias, though the three whose trouble spots were incompletely excised have required antiarrhythmic drugs.

A team of British scientists have employed a similar approach, abetted by a technique called cryothermal mapping, to identify and destroy by freezing sites of electrical irritability and reentrant pathways in 12 patients with arrhythmias that had proved resistant to drug therapy.

Cryothermal mapping was usually employed when the trouble site had been tentatively identified by electrographic mapping. In several patients, however, cryothermal mapping identified these sites when electrographic mapping had failed. It entailed using a cryoprobe to reduce the temperature of the suspected trouble site to between 0°C and -10°C for 15 to 30 seconds. This produced temporary electrical inactivation of the irritable tissues without inflicting permanent damage and thus permitted assessment of the effects on the arrhythmia of taking out that site before irrevocable measures were taken. If temporary inactivation of the site terminated the arrhythmia, the site was permanently ablated by cooling it to -65°C for 2 minutes.

Though the procedure was not completely successful in three patients, the other nine experienced no recurrence of their arrhythmia during 4 to 20 months of followup.

In patients with obstructive hypertrophic cardiomyopathy, surgery may be employed to relieve severe obstruction to left ventricular outflow that does not respond adequately to medication, though most patients with this condition can be managed successfully with drugs. Recently, however, NHLBI scientists reported results of surgery in 12 patients who ordinarily would not have been considered surgical candidates, since the condition either had caused minimal impairment or else appeared to be well controlled by drug therapy. What made these patients different was that all had experienced ventricular fibrillation or cardiac arrest and had survived these potentially lethal episodes only because they were witnessed by bystanders who administered cardiopulmonary resuscitation.

Patients with obstructive cardiomyopathy are at increased risk of sudden cardiac death, with ventricular arrhythmias believed to be the precipitating event in most instances. Agents employed in the medical management of the condition--usually propranolol and, recently, the investigational drug verapamil--have antiarrhythmic properties, but experience suggests that their protection may not always be adequate. Noting significant degrees of outflow obstruction in these 12 patients and in view of their harrowing experience with malignant arrhythmias, the NHLBI scientists reasoned that surgical relief of the obstruction, with consequent reduction of the load on the left ventricle, might improve the electrical stability of the heart and, together with maintenance therapy with antiarrhythmic drugs, confer adequate protection against sudden cardiac death.

Among these patients there was one operative death and another patient died suddenly 9 months after operation. Perhaps significantly, this was a patient in whom operation did not provide effective relief of the outflow obstruction. Autopsy also revealed that he had not taken the prescribed antiarrhythmic medication for at least 8 hours before his death. The other patients have remained well for periods up to 6 years after surgery.

NHLBI grantees have employed a permanently implanted radiofrequency pacemaker to control drug-resistant ventricular tachycardia in three patients. Activated briefly by the patient whenever episodes of tachycardia occur, the pacemaker delivers short bursts of pacing pulses to the apex of the right ventricle at rates substantially higher than those produced by the arrhythmia itself. Paradoxically, this burst of impulses slows the heart, apparently by prolonging the refractory period of conduction tissues enough for the heart's own pacemaker to regain control of heartbeat.

Before the pacemakers were implanted, each patient underwent detailed evaluation, including trials of a variety of approved antiarrhythmic drugs and several still-investigational ones, none of which controlled the arryhthmia. After implantation, the devices were tested against spontaneously occurring or induced tachycardia more than 100 times in each patient to insure that the bursts of pacing pulses did not cause acceleration of the tachycardia or induce ventricular fibrillation. In every instance the device terminated the episode without complications.

Over an average of 13.7 months since implantation of the devices, no patient required hospitalization for an arrhythmia-related problem (though one patient subsequently died of lung cancer). During that period the devices successfully terminated 129 episodes of ventricular tachycardia.

Currently undergoing limited clinical trials at Johns Hopkins is an implantable automatic defibrillator designed to protect patients at high risk of sudden cardiac death from recurrent malignant ventricular arrhythmias. The device monitors heart electrical activity and is programmed to recognize ventricular fibrillation and tachyarrhythmias, whereupon it delivers one or more electrical shocks to the heart to restore normal heart rhythm.

The device is about the size of a package of cigarettes and is powered by lithium batteries with a projected life of 3 years or 100 shocks. At the time of the NHLBI grantees' report, the device had been installed in six patients, in whom it had successfully corrected nine episodes of life-threatening arrhythmias.

Intensive coronary care units have dramatically reduced deaths due to arrhythmias among hospitalized heart-attack patients. However they have enjoyed less success in dealing with pump dysfunction, usually stemming from heavy heart muscle damage sustained during the attack and often manifested by severe left ventricular failure or cardiogenic shock.

Various forms of circulatory assistance have been employed in attempts to avert or cope with such circulatory crises. The aim has been to reduce the workload (and hence the oxygen needs) of the damaged heart while simultaneously improving its blood supply and maintaining adequate perfusion of other organs and tissues.

One such technique employs a balloon-tipped catheter that is introduced into the descending aorta via the femoral artery. Subsequently the balloon is inflated and deflated in synchrony with heartbeat. Inflated as the left ventricle relaxes and refills between contractions, the balloon increases arterial pressure during diastole and so augments coronary perfusion. Deflated as the left ventricle contracts, the device reduces the pressure against which the ventricle must work in pumping blood. Experimental and clinical evidence indicates that the reduced cardiac workload and improved coronary perfusion afforded by intra-aortic balloon pumping may salvage portions of heart muscle threatened, but not yet irreversibly damaged by blood deprivation. Though the technique has not been notably successful in reducing mortality among patients already far gone in heart failure or shock when balloon pumping was initiated, some investigators feel that earlier use of the technique, before the onset of severe cardiac dysfunction, could often avert circulatory calamities and save many lives.

But balloon pumping carries certain risks of its own. In a necropsy study among 45 patients who had died after undergoing the procedure, NHLBI pathologists noted a total of 20 complications in 16 of these patients. Most complications were caused by insertion of the device rather than by its being in place and included dissection of the aorta and/or its distal branches, arterial clots or emboli, arterial perforation, limb ischemia, and wound infection at the site of insertion. Complications of balloon pumping have been estimated to occur in fewer than 20 percent of patients who undergo the procedure; however, noting that most of the complications observed at necropsy had been unsuspected or undiagnosed at the time, the investigators opine that clinical evaluations of these complications probably underestimate their frequency.

Despite a recent wave of enthusiasm for blood-vessel dilators in the management of congestive heart failure (more on this later), conventional treatment continues to rely heavily on augmenting the flagging contractility of the failing heart with cardiac stimulants, such as digitalis or catecholamines together with diuretics to counter the fluid retention that often accompanies the disorder. The usefulness of conventional cardiac stimulants may be limited because of restricted modes of administration (catecholamines, for example, are effective only when given intravenously) or because of toxic side effects that often occur with larger doses. The latter include rapid heart rates, arrhythmias, and undesirable increases or decreases in arterial blood pressure.

A promising new cardiac stimulant, amrinone, appears singularly free of such adverse effects. Limited clinical experience indicates that it produces substantial increases in contractility and cardiac output, but neither increases heart rates nor provokes arrhythmias, has only minor effects on systemic blood pressure, and in general exhibits very low toxicity.

In a recently reported study, amrinone was administered intravenously to eight patients whose heart failure had not been adequately controlled by digitalis and diuretics (though all were continued on this regimen during the study). Amrinone produced sustained increases in heart output, reduced left ventricular filling pressure, and caused more pronounced shortening of circumferential heart muscle fibers. That these increases in contractility occurred in fully digitalized patients indicated that amrinone was summoning up residual contractile reserve even in severely failing hearts.

The infusion period was limited to 180 minutes in this study, but there was no evidence of any decrease in responsiveness to the agent, no arrhythmias occurred, heart rate and mean arterial pressure did not change significantly, and no toxic side effects were noted, even at doses as high as 3 mg. per kilo of body weight.

Though given intravenously in this study, amrinone is also effective when given orally, which may make it an effective adjunct to or substitute for digitalis in the long-term management of congestive heart failure--a prospect being investigated by studies in progress. The mode of action of amrinone is not yet known, though it apparently does not involve the same biochemical systems as are thought to mediate the effects of digitalis or catecholamines.

Other pharmacological approaches to congestive heart failure have emphasized reducing the workload of the heart rather than pushing it to greater efforts with cardiac stimulants. Agents investigated have included venodilators, which reduce left ventricular filling pressure (preload) and also help relieve pulmonary congestion; arteriolar dilators, which reduce the pressure against which the left ventricle must work in pumping blood (afterload); "balanced" vasodilators, which dilate both veins and arterioles and so decrease both preload and afterload; and inhibitors of the renin-angiotensin system, which is thought to contribute to the increased vascular resistance frequently seen in congestive heart failure.

Earlier clinical studies with potent, short-acting vasodilators, such as nitroprusside—usually given by continuous IV drip in hospitalized patients with severe, intractable heart failure—showed that such agents could often avert circulatory collapse and stablilize these patients sufficiently so that treatment with more conventional drugs could be resumed on an outpatient basis.

More recent studies have centered on longer-acting vasodilators, suitable for oral use by ambulatory patients, in the long-term management of milder degrees of chronic heart failure.

Two recently reported studies dealt with prazosin, a drug already in clinical use against hypertension, and a newer, chemically related drug, trimazosin.

Prazosin was compared with a placebo in a 2-month trial among 22 patients with severe congestive heart failure. The prazosin-treated group experienced subjective clinical improvement and also exhibited significantly better cardiac function and exercise tolerance than did the placebo group. The beneficial effects of prazosin were more pronounced during exercise than at rest. As drug treatment continued, there was some attenuation of the beneficial effects of prazosin, possibly because of a gradual increase in the activity of the reninangiotensin system and an increase in sodium and fluid retention. Even so, however, the cardiac status of these patients after 2 months was still substantially better than it had been before drug treatment.

Trimazosin was compared with a placebo among 23 patients with chronic, stable heart failure of varying degrees of severity to assess the longterm effects of the drug on exercise tolerance. The drug-treated patients were followed for periods up to 52 weeks. Sustained improvement in exercise capacity and oxygen uptake occurred in all patients with milder degrees of cardiac failure and in six of nine with severe failure. There appeared to be little attenuation of the drug's beneficial effects on exercise tolerance. However, if drug tolerance did develop, it may well have been masked to some extent in these patients because, by permitting them greater physical activity, drug therapy may have contributed to a generally higher state of physical conditioning.

The reduced heart output often associated with congestive heart failure may provide the stimulus for increased release of renin by the kidneys. In the blood, renin acts on a plasma protein to generate angiotensin I, a relatively inactive peptide. However, as it traverses the pulmonary blood vessels, angiotensin I is acted upon by a converting enzyme to generate angiotensin II. This powerful blood vessel constrictor may contribute importantly to the increased vascular resistance which is common in congestive heart failure and which can impose further burdens on an already overtaxed heart.

Captopril, an agent that impedes angiotensin II production by inhibiting the coverting enzyme that generates it from angiotensin I, had previously been shown to reduce blood pressure in hypertension, especially of the "high renin" variety. In the recently reported studies, investigators evaluated captopril in patients with congestive heart failure, some of whom has not responded well to therapy with other vasodilators. In all patients, captopril improved left ventricular function and increased heart output, which resulted in clinical improvement and increased exercise tolerance. In patients placed on maintenance therapy with captopril, the drug's beneficial effects persisted over followup periods ranging from 2 to 7 months, during which there was no evidence of diminished responsiveness to the agent. The investigators concluded that captopril may be a useful addition to the armamentarium for the long-term management of congestive heart failure. It may be especially useful in combination with certain other vasodilators whose long-term effectiveness might otherwise be blunted by their stimulation of the renin-angiotensin system, though this remains to be demonstrated.

The Reis-Hancock prosthetic heart valve of animal origin (the pig) has been highly satisfactory for replacement of diseased aortic, tricuspid and mitral valves. Mitral replacement seems to be the more successful than aortic replacement probably due to different blood pressures on the valves. The Hancock valve combines good performance characteristics with exceptionally low risk of clots or emboli, thus obviating the need for anticoagulants postoperatively. The unanswered questions about this valve, as with bioprosthetic valves in general, had concerned its durability.

It would apppear from various studies that the useful life of the prosthetic valve is typically between 5 and 8 years and in many patients, especially children, it may be much shorter. Calcification appears to develop more frequently and at an earlier stage after insertion in children than in adults.

Examination of failed valves revealed calcification or disruption of the valve leaflets. Microscopic examination showed degeneration of their collagen fibrils, calcification and infiltration by lipid and fibrinoid substances.

An NHLBI grantee working on a phonocardiographic technique for the early detection of aortic valvular stenosis found that by analyzing the A_2 (second heart sound) the cardiologist may get an early indication of increased stiffness of the aortic valve leaflets of the prosthetic valve. The study showed that the A_2 in the normal aortic valve was 53 ± 3 hertz, but was substantially higher (87 ± 5 hertz) in those with calcific aortic stenosis. Patients with an aortic Hancock valve implanted for less than 18 months had an A_2 frequency equivalent to those with normal valves. However, patients with valves in place 5 years or longer had frequencies within the range of patients with calcific aortic stenosis.

Several patients in this series required replacement of their implanted Reis-Hancock valve because of valve failure. All had exhibited the higher frequency ranges indicative of aortic stenosis.

A recent NHLBI-supported study may provide a clue to why pig valves fail so often and so early in younger subjects. Over a 5-year period, the investigators implanted 47 of these valves in 44 patients under 20 years of age. Eight of the valves failed, beginning 22 months after implantation, and had to be replaced. All showed calcification, and in most it was the primary cause of valve failure. Although the numbers were small, the site of implantation--whether in the aortic, mitral, or tricuspid positions--seemed to have little bearing on whether the implant subsequently became calcified.

The investigators noted that calcified valves contained unusually high levels of protein-bound gamma-carboxyglutamic acid, an amino acid with a high affinity for calcium. The amino acid was not found in untreated porcine valves nor in "cured" prostheses prior to implantation. Apparently, gamma-carboxyglutamic acid was deposited into the valve after implantation and may have been followed by movement of calcium into the valve leaflets. Suspected as the carriers of the amino acid are macrophages, "scavenger cells" that are usually attracted in large numbers to sites of infection, inflammation, or wherever "foreign" substances have gained access to the body. Why gamma-carboxyglutamic is apparently deposited more rapidly in younger valve recipients than in older ones is not yet clear.

Gamma-carboxyglutamic acid requires vitamin K as a cofactor in its synthesis, as do several blood clotting factors. This suggests that vitamin K antagonists, such as coumadin-type anticoagulants, might be of value in preventing premature calcification of implants in younger subjects. Ironically, however, the principal reason for choosing the pig valve over other prostheses has been that the pig valve was much less likely to cause thromboembolic problems and rarely required postoperative anticoagulation.

For some patients with hopelessly damaged hearts, heart transplantation may offer a therapy of last resort. Ideally, the candidate for this procedure should be less than 50-years old; he should not have pulmonary hypertension, unresolved pulmonary blood clots, or pulmonary infarction; he should be free of any serious infection; and he should not have insulindependent diabetes.

Better criteria for selecting patients most likely to benefit from heart transplantation, improved techniques for early detection of rejection episodes and for monitoring immunosuppressive therapy, early and aggressive diagnosis and treatment of infections, and careful control of diet and use of drugs designed to diminish the high vulnerability of the transplant to accelerated coronary arteriosclerosis: all of these have contributed to the success of the heart transplantation program at Stanford.

Currently, 65 percent of transplant recipients of Stanford survive for a year or more, with about 50 percent surviving for 5 years or more. The great majority of long-term survivors are restored to normal cardiac functional status by their transplants and, if they wish, can resume their previous occupations.

The intensive steroid therapy sometimes required to protect the transplant against rejection also reduces the patient's resistance to infections, currently the main hazard to survival. Although the frequency of both rejection episodes and infections usually diminishes markedly after the first postoperative year, morbidity and mortality associated with chronic immunosuppression remains a significant problem.

A new immunosuppressive agent called cyclosporin A may help to alleviate some of these difficulties. Produced by a fungus called Trichoderma polysporum, cyclosporin A was initially screened for antibacterial action, which it lacked; but it did exhibit an immunosuppressive action apparently more specific than that of steroids. Evidence suggests that cyclosporin A attacks primarily "activated" lymmphocytes (T-cells)--usually the "shock troops" in the assault on the graft during rejection episodes--whereas steroids go at various elements of the immunological system more or less indiscriminately. Steroids may also produce profound, sometimes troublesome effects on fluid and electrolyte balance or on other aspects of metabolism.

Cyclosporin A also has side effects. These include kidney toxicity and, and, probably, an increase in treated patients' risk of malignant lymphoma (which also occurs with steroid therapy but apparently less often). As a result, though cyclosporin A has been the only immunosuppressive agent used in a few studies, most investigators feel that it is best used in conjunction with steroids, thereby reducing dosage requirements for each agent.

Cyclosporin A, with or without steroids, has been undergoing clinical testing in conjunction with kidney, liver, and bone marrow transplants--with generally promising results. It has not been tried clinically in heart transplant recipients; but the Stanford group plans to test the drug in conjunction with steroids to see if the combination will help eliminate the "steroid backlash" that has been a persistent problem in this and other organ transplantation programs.

Patients admitted to the Stanford transplantation program but awaiting donor hearts have a life expectancy without transplantation of only a few months. Some have succumbed before donor hearts could be obtained in the immediate area. Accordingly, Stanford investigators looked into the possibility of procuring donor hearts from more distant locations, especially large urban areas where noncardiac deaths from accidents and the like are relatively frequent. They also sought simple but effective means for harvesting and preserving those hearts to sustain their viability and function during shipment from donor to prospective recipient.

Between June 1977 and November 1979, 34 donor hearts, transported to Stanford from as far as 900 km away, were implanted in patients. Survival among these recipients (63 ± 8%) did not differ significantly from that of patients receiving donor hearts obtained locally.

Distantly procured hearts were harvested after inducing cardioplegia with infusions of a cold (4°C) solution containing electrolytes, dextrose, and mannitol. The hearts were then placed in sterile plastic bags and immersed in a sterile cannister filled with saline. The cannisters were packed in ice for transportation to Stanford, where the hearts were implanted as soon as possible after arrival. The distantly procured hearts were bloodless for periods of 113 to 192 minutes, versus 39 to 75 minutes for hearts obtained locally.

The investigators compared ultrastructural changes in biopsy specimens obtained before and after reperfusion in distantly obtained versus locally obtained hearts. The former showed more profound alterations in cell nuclei, mitochondria, endothelium, and muscle fibers, as might be expected from their longer periods of blood deprivation. However, the hearts functioned normally after implantation and, postoperatively, the recipients fared just as well as did recipients of hearts obtained locally. Biopsies obtained after 1 year showed that the earlier ultrastructural differences between distantly obtained and locally obtained hearts were no longer discernible.

From time to time, lung transplants have been attempted in patients moribund from advanced lung disease. The results have been disappointing: the longest survivor (one patient) lasted only 10 months, and most recipients succumbed within hours to days of receiving their transplants.

However, the results of animal studies at Stanford raise the possibility that combined transplantation of the heart and both lungs may become clinically feasible in the near future. If so, the perfected procedure might find application in patients suffering from advanced bilateral lung disease, with or without heart failure, or in patients with primary heart disease complicated by secondary pulmonary vascular problems, as occurs with some kinds of congenital defects of the heart and great vessels.

The investigators performed the combined procedure, employing two different operative techniques, in 27 monkeys. Some of the animals received autografts--that is, their own heart and lungs were removed, then reimplanted--whereas others received allografts: hearts and lungs from unrelated donor animals.

In 17 animals, the tranplantation procedure was done under deep hypothermia and circulatory arrest. Allograft recipients received no immunosuppressive therapy post operatively. Only one animal--an autograft recipient--was a long-term survivor; all allograft recipients died within 5 days.

In 10 other animals the procedure was done with heart-lung bypass. All allograft recipients were subsequently treated with immunosuppressive agents: azothioprine (for the first 14 days only) and Cyclosporin A (begun at higher doses and then gradually reduced to daily "maintenance doses" that were continued indefinitely).

Collectively, these animals fared much better than did those receiving their transplants under hypothermia and circulatory arrest. With heart-lung bypass, all autograft recipients were long-term survivors as were three of seven allograft recipients. Of the latter, one succumbed after 144 days to lymphoma, whereas the other two were still alive and well at the time of the grantees' report (276 and 311 days after surgery, respectively). At that time, there was only minimal evidence of rejection episodes and there had been no significant problems with infection.

The investigators noted that the substantial Stanford experience with heart transplantation and its associated problems is probably directly applicable to heart plus lung transplants as well. They further opined that cyclosporin-A may prove to be of considerable value in the prevention and control of rejection problems.

Lung Diseases

The lung damage of emphysema is thought by many investigators to ensue from local imbalances in lung between enzymes called proteases, which are capable of attacking the collagen and elastin of lung connective tissue, and antiproteases that normally neutralize them.

The source of these proteases are various species of white blood cells, particularly neutrophils and alveolar macrophages, which release these potentially lung-damaging enzymes in the course of their encounters with bacteria, inhaled particulate matter, and other "foreign" invaders of the lung.

Chief among the protective antiproteases is alpha-1-antitrypsin. This antiprotease is mainly effective against enzymes that attack elastin, the connective tissue component thought to sustain the most damage in emphysema. A very small fraction of the population (about 1 in 1,500) suffers from a severe, genetically determined deficiency of alpha-1-antitrypsin, with levels only about one-ninth normal or less. Homozygous individuals, who inherited the defective gene from both parents, are highly vulnerable to emphysema, which usually develops earlier and progresses more rapidly in these subjects than in those who do not have the genetic trait or else are heterozygous for it.

Investigating means of increasing lung protection against elastases in homozygous individuals, NHLBI investigators found that 4 grams of concentrated alpha-1-antitrypsin, obtained from normal plasma and administered intraveneously once a week, maintained "protective" levels of the antiprotease (80 mg/dl or more) over the 4 weeks of the study. They also found that oral administration of danazol, a testosterone-like drug previously used chiefly in the treatment of endometriosis, raised blood levels of "native" alpha-1-antitrypsin by about 50 percent in homozygotes, but not to levels believed necessary for adequate lung protection.

Other research has indicated that the amino acid methionine occupies a critical position in the active site of the alpha-1-antitrypsin molecule. In fact, several methionine residues appear to be involved. Oxidation of one or two methionine residues to methionine sulfoxide abolishes the enzyme's inhibitory activity against elastase and, with oxidation of additional methionine residues, its activity against trypsin and chymotrypsin is lost as well. The extent of enzyme inactivation appears to be proportional to concentration of the oxidant and the duration of the exposure.

Agents to which individuals may be exposed and which are capable of oxidizing the methionine of alpha-1-antrypsin include ozone and certain components of photochemical smog and cigarette smoke. Thus, reducing the protection of the lung against elastases may be one mechanism by which smoking and exposure to air pollutants increase the individual's risk of emphysema.

There has long been abundant evidence concerning the harmful effects of smoking on the heart and lungs of the smoker himself. Until recently, however, involuntary exposure of nonsmokers to the smoke of others had been considered more a nuisance than a health hazard. But studies done during recent years indicate that exposure of nonsmokers to ambient cigarette smoke results in significant levels of nicotine in blood and urine, can aggravate symptoms in subjects with angina or peripheral vascular disorders, and can provoke respiratory symptoms in individuals highly sensitive to various irritants in cigarette smoke. Moreover, young children of smoking parents appear to have more respiratory complaints than do children of nonsmoking parents.

Most recently, investigators employed spirometry to evaluate function of the small airways of the lungs in 1) nonsmokers not exposed to cigarette smoke on the job, 2) nonsmokers chronically exposed for 20 years or more to the smoke of co-workers, and 3) long-term voluntary smokers.

As expected, nonsmokers working in smoke-free environments scored highest on all spirometric tests and heavy smokers the lowest. A disturbing finding was that nonsmokers chronically exposed on the job to the smoke of others exhibited significant deterioration of small-airways function as a result of that exposure. Their spirometric scores indicated at least a 10 percent decrease in functional capacity of the airways: about the same as that of smokers who do not inhale or those who consume up to half a pack a day and inhale the smoke.

The problem of the nonsmoker working in facilities that permit smoking on the job is likely to grow worse rather than better, for indoor air quality could deteriorate further as concerns over energy conservation place stricter limits on the amount or quality of ventilation provided in the workplace.

The lung contains no anticollagenese. While collagen is relatively immune to most proteases, the neutrophil produces a collagenase capable of attacking type I collagen, an important connective-tissue component of the gas exchange units (alveoli) as well as of the larger airways and blood vessels of the lung. Formerly, this was thought to be the only one of the four types of collagen found in lung that was vulnerable to degradation by proteases. But NHLBI studies indicate that an elastase produced by neutrophils, though showing some preference for elastin, is also capable of degrading type III collagen, which is found in the same lung structures as type I, but is only half as abundant. Thus it appears that elastases, thought to be the principal perpetrators of lung damage in emphysema, can, unless neutralized, chew up two of the main connective-tissue components of the lung airways and alveoli.

Other NHLBI-supported studies are investigating synthetic substances of low molecular weight that exhibit antielastase activity. One promising group of inhibitors, called peptide chloromethyl ketones, have been shown to protect laboratory animals against the lung damage that usually follows airway instillation of elastases. They appear most effective when incorporated into albumin micropheres that, after injection into the blood, facilitate the accumulation and retention of the inhibitors in lung.

Another study showed that these inhibitors could protect the animals' lungs against instilled elastases when administered orally, but only if given very shortly before the protease challenge. The investigators suspect that the protection conferred by the inhibitors was brief because of their fairly high nonspecific reactivity. They will combine, not only with elastase, but with other proteins containing sulfhydryl (-SH) groups. Thus much of the inhibitor reaching the lungs may soon become bound to connective tissue substrates and hence unavailable for neutralization of elastase.

The interstitial lung diseases account for about 15 to 30 percent of non-infectious lung conditions. They are characterized by inflammation of the lung gas-exchange units (alveoli), degradation of connective tissue collagen, progressive distortion of alveolar-capillary units, and formation of nodules (granulomas) or scar tissue in affected areas of the lung. The diseases are accompanied by the accumulation in lung of various species of inflammatory and immune effector cells, which are thought to be mediators in the ensuing lung damage.

In one of these disorders, sarcoidosis, activation of the cell-mediated immune system is thought to be involved in granuloma formation during active stages of the disease; yet skin and blood tests may suggest depression of cellular immunity. For example, monocytes are believed to be the chief building blocks in granuloma formation; yet monocytes in peripheral blood of patients with sarcoidosis may exhibit muted response to antigens or mitogens that usually induce vigorous proliferation. Similarly, T-lymphocytes are believed to be key participants in the alveolitis and granuloma formation of sarcoidosis formation; yet the levels of these cells in the peripheral blood of sarcoidosis patients are often abnormally low.

However, NHLBI studies indicated that in sarcoidosis the immunological response is localized at sites of disease activity, so that peripheral blood samples may provide no index of the intensity of that response in affected organs.

The investigators examined T-lymphocytes in peripheral blood, in lung lavage fluid, and in bone marrow obtained from patients with sarcoidosis, from patients with lung fibrosis of unknown origin, and from normal subjects. The patients with sarcoidosis exhibited abnormally low levels of T-lymphocytes in peripheral blood, but greatly elevated levels in their lung lavage fluid. A high proportion of these cells in lavage fluid appeared to be activated. In contrast, there were no differences between blood and lavage fluid levels of T-lymphocytes in normal subjects and in patients with pulmonary fibrosis, nor were any of these lymphocytes activated.

In sarcoidosis patients, T-lymphocyte levels were normal in uninvolved bone marrow, but were elevated in areas where granulomas were present.

The results indicate that the activated T-lymphocyte is indeed involved in the alveolar inflammation and granuloma formation of sarcoidosis, though this participation may not be evident outside the involved tissues themselves.

Sarcoidosis may be difficult to diagnose during its early stages. Usually the diagnosis is established by demonstrating granulomas in tissue biopsies from patients with clinical findings suggestive of sarcoidosis and by the exclusion of other diseases that may produce a somewhat similar clinical or morphologic picture.

Several years ago, NHLBI grantees showed that the activity of angiotensin converting enzyme was often elevated in the serum of patients wih sarcoidosis but was normal in patients with disorders that might sometimes be confused with sarcoidosis. Their results suggested that determination of converting enzyme activity could be highly useful in differential diagnosis.

Recently, other scientists developed a simple radioassay procedure for determining converting enzyme activity that appears to offer certain advantages over spectrophotometric and spectrofluorometric assay procedures used earlier. They tested it among patients with "active" sarcoidosis, among those with arrested or cured sarcoidosis, and among subjects with other pulmonary disorders.

In general, though there were a few false positives and a few false negatives, the activity of angiotensin-converting enzyme 1) was elevated in patients with active sarcoidosis, 2) was normal in patients with arrested or cured sarcoidosis or who had other lung disorders, and 3) decreased toward normal among patients with active sarcoidosis who were receiving effective treatment with steroids. The results indicate that measurements of angiotensin-converting enzyme may be useful both in the diagnosis of sarcoidosis and also in the evaluation of therapeutic measures.

Blood Diseases

Factor VIII concentrates have greatly improved the management of bleeding episodes in hemophilia. But some hemophiliacs develop antibodies to factor VIII. Those antibodies neutralize much of the factor VIII infused into these patients and so can make the control of bleeding extremely difficult.

One approach to circumventing these antibodies has been to employ prothrombin-complex concentrates, thus bypassing the factor VIII-requiring step in coagulation. In a recent NHLBI-supported trial among hemophiliacs with antibodies to factor VIII, prothrombin-complex concentrates were compared with a placebo in the treatment of joint hemorrhages. On the basis of the patients' subjective response plus objective evidence of improvement in joint mobility after 6 hours, the prothrombin-complex concentrates clearly outperformed the placebo, but were judged effective only about half the time. However, though the concentrates are expensive and only partially effective, the investigators noted that their use to treat joint bleeding in hemophiliacs with factor VIII antibodies is justified in the absence of any other effective and readily available therapy.

At the opposite pole from hemophilia and related hemorrhagic disorders are thromboembolic complications of heart and blood vessel diseases. Two such complications--deep-vein thrombosis and pulmonary embolism--are usually treated with anticoagulants, such as heparin; but at a recent consensus development conference at NIH, the panel noted that the fibrinolytic agents urokinase or streptokinase are safe and effective if the patients are properly chosen (specifically, when the patients are treated shortly after the thromboembolic episode) and when used in conjunction with anticoagulants.

In pulmonary embolism, fibrinolytic therapy usually reduces pulmonary hypertension, normalizes hemodynamic responses, and helps prevent permanent damage to the pulmonary vascular bed. In deep-vein thrombosis, fibrinolytic therapy helps prevent permanent damage to the venous valves. Though serious bleeding had been a complication of fibrinolytic therapy in earlier clinical trials, the panel noted that it is a lesser risk than had previously been thought. Bleeding is easily controllable if invasive procedures are kept to a minimum and patients are carefully selected and monitored.

The panel concluded that, properly used, fibrinolytic therapy represents a significant advance in the management of proximal deep-vein thrombosis and the more severe forms of pulmonary embolism.

In research concerned with sickle cell disease, the quest continues for safe, effective agents that will inhibit or reverse sickling and thus prevent sickle cell crises or else reduce their severity.

A group of compounds that may have promise as antisickling agents (though none has yet been tested clinically) are benzyl esters of amino acids. Preliminary studies of these esters have shown that they inhibit sickling of red cells in vitro, apparently have low toxicity, and do not interfere with the oxygen-carrying function of red cells.

Other agents that may be useful in the treatment of sickle cell disease are inhibitors of calmodulin, an intracellular protein that acts as a receptor for calcium. Normal red cells do not take up calcium, but the red cells of those subjects with sickle cell disease are abnormally permeable to this ion. Calcium influx into these cells is enhanced by deoxygenation and sickling. In the cell this calcium is thought to interact with calmodulin and perhaps other proteins of the cell membrane to "fix" the membrane permanently in the sickled conformation. Irreversibly sickled cells, which may constitute 5 to 25 percent of all red cells in victims of sickle cell disease, are usually culled rapidly from the circulation and destroyed.

Earlier studies indicated that calcium influx into red cells was partially inhibited by zinc. More recent studies indicate that zinc plus thioridazine, a calmodulin inhibitor and tranquilizer, appears to be even more effective. In limited clinical trials the combination reduced the rate of hemolysis and substantially increased the half-life of sickled cells.

A possibly important factor in sickle cell crises may be the tendency of red cells of sickle cell disease patients to adhere to the epithelium of blood vessels. A recent study showed that whereas red cells from normal subjects would not stick to umbilical epithelium, some younger, reversibly sickled cells from sickle cell disease patients and a major proportion of their older irreversibly sickled cells clung tenaciously to epithelium through numerous washings.

The investigator thinks that crisis episodes may develop when the affinity of the patients' red cells for vascular endothelium slows bloodflow in smaller vessels sufficiently to cause the cells to become deoxygenated and then to sickle in these vessels, which they subsequently block. He believes that agents capable of reducing the stickiness of red cells in sickle cell disease might be effective in preventing crisis episodes.

Patients with Cooley's anemia require repeated blood transfusions to keep their levels of red blood cells within tolerable limits. Each transfusion introduces substantial amounts of iron, which the body cannot excrete readily and which it tolerates poorly. Eventually excess iron accumulates in various tissues and organs, including the heart. Cardiac complications of this iron overload, chiefly arrhythmias or congestive heart failure, are presently the leading causes of death among victims of Cooley's anemia.

The normal red blood cell has a lifespan of about 120 days. Patients given conventional transfusion therapy receive a mix of red cells of varying ages--some young, others "middle aged," and still others already nearing the end of their useful life when first infused. However, scientists at NIH and at Boston Children's Hospital have developed a novel transfusion scheme whereby their patients with Cooley's anemia receive only young red cells. By means of an automatic cell separator, young red cells are separated from the older cells at the time of donation, the young cells being retained for subsequent transfusion use, the older cells being immediately returned to the donor.

Because the young cells should persist longer in the circulation after infusion, the investigators feel that the technique will reduce by half the number and frequency of transfusions required. This, in turn, would significantly reduce the iron burden imposed. Although currently being evaluated in patients with Cooley's anemia, the new procedure should also be of benefit in severe sickle cell disease, aplastic anemia, or other conditions requiring transfusion therapy.

To combat the iron overloading that occurs in Cooley's and other anemias requiring transfusion therapy, investigators have been seeking effective chelating agents, which combine with iron compounds to form complexes more readily excreted by the kidneys. One of the best iron chelators is desferrioxamine. However, its effectiveness has been limited by its rapid excretion after infusion, much of it being cleared before it has chelated any iron.

Earlier studies had demonstrated that slow subcutaneous administration of desferrioxamine by means of a small clockwork pump enhanced the ironchelating effectiveness of the drug. More recently, investigators have found that incorporating the drug into red cell ghosts, which were then administered by slow intravenous infusion, made the drug still more effective.

The red cell ghosts employed as drug carriers in this study were essentially membranous sacs from which most of the original contents had been removed and replaced by desferrioxamine, after which the membranes were chemically "resealed" prior to infusion. Though the drug-laden ghosts are themselves cleared fairly rapidly from the circulation, they persist longer than the free drug. Moreover, they are culled from the circulation by reticuloendothelial cells--themselves a major repository of excess iron--and so continue chelating to the very end.

Compared with subcutaneously administered desferrioxamine, the drug-laden ghosts more than doubled the patients' iron excretion (mean increase: 119 percent; range 7 to 379 percent). In some patients with very severe iron overloading, urinary iron excretion approached the theoretical maximum possible with the doses of desferrioxamine administered. Also noteworthy were the high rates of iron excretion achieved among patients with relatively modest iron overloads. Such patients usually respond poorly to chelation therapy, and it is in such patients that the investigators feel the new technique will be most useful.

The epidemiologic studies of NHLBI grantees have provided most of our current knowledge concerning the incidence and mode of transmission of hepatitis viruses. Identification of high-risk population groups has established the groundwork for our understanding of the spread of hepatitis and was necessary before clinical trials using newly developed hepatitis B vaccines could be mounted.

Viral hepatitis is still the most serious complication of blood transfusion. Posttransfusion hepatitis develops in 7 to 10 percent of blood recipients. Ninety percent of this hepatitis is of a type called non-A, non-B (NANB) hepatitis. The reality of NANB hepatitis is now firmly established both epidemiologically and clinically. Serologic screening, which has proved so successful in the control of hepatitis B, will not be feasible until the virus(es) responsible for NANB hepatitis are identified. However, an NHLBI-sponsored collaborative study has shown that donor blood containing elevated levels of serum transaminase (ALT) carries a high risk for NANB hepatitis. Screening blood donor samples for elevated ALT levels may be useful as an interim assay for reducing the incidence of posttransfusion hepatitis.

The NHLBI continues to support the development of oxygen-carrying blood substitutes. The first transfusions of a perfluorochemical blood substitute into humans in the United States occurred this year. Blood substitutes offer great promise both in chemical applications, particularly in emergency situations when adequate supplies of blood are not available, and in research applications, particularly relative to the kinetics of blood cell and plasma protein development.

Division of Heart
& Vascular Dis.

DIVISION OF HEART AND VASCULAR DISEASES
ANNUAL REPORT

General Mission

A. The Division of Heart and Vascular Diseases has continued the broad mandated responsibilities established in 1948 by the National Heart Act which created the National Heart Institute, the predecessor of the National Heart, Lung, and Blood Institute, as the primary federal agency responsible for the conduct and support of research relating to the cause, prevention, methods of diagnosis and treatment of diseases of the heart and blood vessels.

B. Additionally, under Public Law 95-622 the extension of authorization for research training through National Research Service Awards, the Division has authority to make grants for pre- and post-doctoral training of individuals to undertake research in the causes, diagnosis, prevention and treatment of cardiovascular diseases.

Magnitude of the Problem

C. In 1977 three of the ten leading causes of death in the United States were problems that fall with DHVD's purview: diseases of the heart (ranked first), cerebrovascular diseases (third), and arteriosclerosis (eighth). Although the problem is enormous, significant advances are being made. Since 1968 there has been a steep decline in cardiovascular death rates. Deaths from coronary heart disease have declined by over 22 percent in the last 10 years, and deaths from stroke have decreased by over 33 percent since 1968.

While it is not known which factors have been most responsible for this decline, improved medical practices, more widespread treatment of high blood pressure reduction of cigarette smoking and a possible reduction of blood cholesterol concentrations in the general population through reductions of saturated fat in the diet are all probable contributors to this improvement in health status.

Despite striking improvements, however, cardiovascular disease remains among the Nation's most serious health problems. It constitutes the leading cause of death for men beginning at age 40 years and for women beginning at age 60. At least 40 million Americans have diseases of the heart and blood vessels.

Progress Toward Achieving Objectives

In the past year considerable progress has taken place in the programs of the Division. The Division has maintained its strong base of research support for investigations into the causes of heart and vascular disease including research in prevention and in improved therapies. High priority also has been given to provide training for promising young investigators including minority professionals to continue manpower development for research in cardiovascular diseases. Three new training programs were funded: 1) clinical investigator awards, 2) senior fellowship, 3) short term training. A committee from the Training Centers produced a report for trainees, Handbook on Hypertension.

Six new initiatives were implemented:

1. NHLBI Registry for Percutaneous Transluminal Coronary Angioplasty
2. Methodology for Estimation of Human Dietary Sodium Intake/Excreti
3. Changes in Pathology of Arteriosclerosis and Myocardial Infarctio over time since 1960.
4. The Role of the Microcirculation in Hypertension
5. Standardization Program for HDL-Cholesterol Measurements
6. Dahl S/R Hypertensive Rat Colony

Thirteen new research initiatives recommended by the Division and its Advisory Committees could not be undertaken with the funds available for FY 1980.

Highlights of Accomplishments

The Aspirin-Myocardial Infarction Study (AMIS)

A randomized, double-blind, placebo-controlled clinical trial was initiated in 1975 to evaluate the efficacy of aspirin in the secondary prevention of coronary heart disease. Thirty clinical centers recruited 4,524 patients within a thirteen-month period with a three-year follow-up. Results of AMIS were reported in JAMA 243:661-669, 1980. No statistically significant differences in all cause mortality of coronary heart disease incidents were found.

Hypertension Detection and Follow-Up Program (HDFP)

Pilot studies for this program were initiated in 1971. Fourteen clinics recruited 10,940 black and white men and women into this randomized, controlled cooperative clinical trial. Results of HDFP were released in November 1979 and appeared in JAMA 242:2562-2577, 1979. Systematic management of hypertension at the HDFP clinics resulted in a 16.9% reduction in five-year mortality compared to the group who received the usual sources of community care. Mild hypertensives, i.e. 90-104 mm Hg treated at the HDFP clinics, experienced a 20.3% reduction in mortality.

Lipid Research Clinics Program (LRC)

This program concentrates on the detection, prevalence, and treatment of lipid disorders. Reliable distributions for HDL-cholesterol levels are now available for the North American population. Recent analysis of the ongoing Mortality Follow-up Study data confirm in a prospective manner the inverse relationship between HDL cholesterol and risk of death from cardiovascular disease in a population primarily over 50 years of age. Genetic and pediatric studies have demonstrated progress in the identification of hypercholesterolemic and hypertriglyceridemic children, and the role of nutrient intake to lipids and lipoproteins in children.

Coronary Artery Surgery Study (CASS)

A collaborative randomized national effort was initiated in 1973 to study in detail the indications for coronary artery surgery and the true, long-term benefits of such surgery compared to medical management. Recruitment of 780 patients ended in June 1979; in addition 24,188 patients were enrolled in the CASS Registry. All patients will be followed for five years. Two reports were released in the past year from registry studies on the complications of coronary arteriography and on the results of exercise stress testing.

High Blood Pressure Education Program (HBPEP)

The national impact of this program continues to be felt. Important trends related to the program include the following:

a. From 1968 to 1977, age adjusted death rates from hypertensive disease, coronary heart disease, and stroke declined 51%, 23%, and 32% respectively.

b. From 1971 through 1978, physician visits for hypertensive disease increased 20.7%, while visits for all causes decreased 10.2% during the same period.

c. Two recent national surveys indicate public knowledge of hypertensive disease is improving.

HDFP findings are being evaluated for expanded usefulness to the professional and lay communities. The role and importance of dietary management is increasing, and Blue Cross continues its nationwide program to train account executives in marketing worksite control programs.

Atherogenesis

New information is now available on the regulation of cholesterol synthesis by endothelial cells. The activity of high affinity receptors which occupy 5% of the cell surface and permit internalization of neutral lipid without uptake of chylomicron protein has been observed. Cholesterol entering the cell in this manner is highly effective in the regulation of cellular cholesterol and maintenance of cellular integrity.

The mechanisms responsible for the excessive deposition of cholesterol in tissues and arteries of individuals with homozygous familial hypercholesterolemia has been reported from studies in mouse peritoneal macrophages. Results indicate the existence of binding sites on macrophages for modified lipoproteins. This mechanism does not appear to be under feedback regulation and may help explain the development of macrophages into foam cells in atherosclerotic plaques.

A new human apoprotein, A-IV, has been isolated and characterized. It is secreted by the intestine and its concentration in plasma rises after fat feeding. Detectable amounts are found in HDL separated by gel filtration chromatography. Its physiological role remains to be defined.

Behavioral Medicine

Over the past year several grant projects produced data supporting the ability of stress to induce renal tubular retention of water and sodium and to modulate baroreceptor reactivity. A report has been prepared by a panel of experts summarizing the state of the art/compliance promotion methodology, research target areas for the '80's, and the results of the Institute's grant-supported Education Research Program. Projects that address the physiologic basis for Type A coronary heart disease association have demonstrated considerable specificity of behavioral and cardiovascular response characteristics in individuals designated Type A by the Structured Interview. A major factor in prediciting cardiovascular arousal (blood pressure and heart rate elevations and increased plasma epinephrine) is the degree of perceived social or performance challenge present in the testing situation. Type A's react to challenges such as competition, hostility, and time pressure with greater and longer lasting cardiovascular arousal than Type B's under similar situations.

Epidemiology and Biometry

Results from a ten-year follow-up of 387 working women and 350 housewives from the Framingham Heart Study demonstrated that employment by women, per se, was not a significant risk factor for coronary heart disease. Sub-group analysis, however, suggested that the stress of some working environments, plus the burdens of raising a family, may be associated with the development of CHD in women.

A genetic study of 200 families having a child with homocystinuria compared members of control families (those with a child with a new mutation achondroplastic dwarf or with impaired phenylalanine metabolism) and found no statistically significant increases in heart attacks or strokes for relatives of homocystinuric patients.

It is apparent in the High Blood Pressure in the Young Program that the most significant correlates of blood pressure in children are body weight and changes in body weight. Several of the studies confirmed a consistent relationship between maternal blood pressure and blood pressure in children, indicating the likelihood of a genetic factor operating early in life. These results have considerable implications for identifying those individuals, early in life, who might be most prone to develop hypertension later on in life.

Circulatory Assistance

Presently, assist devices are being designed to provide 10 liter per minute blood flow at 120 beats per minute against a peak arterial pressure of 150 mm Hg with a filling pressure of 15 mm Hg. Pumps suitable for long-term use, and with mechanical rather than pneumatic actuation, are in various stages of development and testing. Three long-term ventricular assist blood pumps have operated in calves for up to six months without anticoagulation. All three can be implanted without systematic heparinization or the use of cardiopulmonary bypass.

Biomaterials

Recently, investigators have begun to assess the role of trace proteins such as fibronectin, antithrombin III, or complement (C3) which may profoundly affect coagulation or thrombosis. Plasma lipid studies suggest that in a blood-material interaction they act as a nidus for calcification or react with platelets as potential prostaglandin precursors.

Black Health Providers Task Force

In June 1979, the Task Force ended its work with the announcement of recommendations for suggested roles in high blood pressure control for six provider groups (physicians, dentists, nurses, pharmacists, optometrists and pediatrics), as well as recommendations which addressed barriers to the performance of these roles. Final, written recommendations from the Task Force are now available.

Significant Workshops

1. Percutaneous Transluminal Coronary Angioplasty (PTCA).

   This workshop concluded that the technique is most applicable to a small subset of patients with recent onset of symptomatic ischemic heart disease and a proximal obstruction in a single coronary artery. The workshop participants agreed that PTCA is an investigational technique and that skilled angiographers need specific training for the procedure and a close working relationship with a surgical team that can provide standby operative capability.

2. Improving Clinical and Consumer Use of Blood Pressure Measuring Devices

   This NIH Consensus Development Conference brought together representatives of the medical and engineering professions, consumer groups, and government in an effort to help develop national voluntary guidelines for use and manufacture of sphygmomanometers.

3. Use of a Platelet Active Drugs in the Secondary Prevention of Cardiovascular Events

4. Cholesterol and Non-Cardiovascular Disease

5. Noninvasive Techniques for Assessment of Atherosclerosis in Peripheral, Carotid and Coronary Arteries

   This workshop looked at clinical usefulness of noninvasive methods for imaging plaques in arteries and then following the natural history.

6. Needs and Opportunities for NHLBI Activities on Sudden Cardiac Death

   The prevention of sudden cardiac death in persons with atherosclerotic heart disease was the target of this workshop.

7. Ambulatory ECG Recording

   This workshop discussed the state of the art EKG ambulatory monitoring, and made recommendations for future research.

Major Problem Areas

The lack of a permanent Director and insufficient positions for needed staff are still the major problems of the Division.

The limitation of extramural research support funds is of major concern.

The reduction in the number of research grants that can be supported will result in disbandment of established research teams and loss of needed research.

Uncertainty with regard to the FY 1981 training budget, when currently the commitments are in excess of the funds in the budget, is worrisome.

The travel allocation to the Division, both domestic and foreign, is not only decreasing, but reductions are announced after travel plans have been arranged. At the same time costs are rising precipitously: there have been four air-fare increases in the past six months.

Difficulties with staff promotions beyond certain levels and decreased flexibility with regard to recruiting into certain types of positions and series are both demoralizing and demotivating. The personnel freeze is an additional restriction on personnel actions.

Division of
Lung Diseases

DIVISION OF LUNG DISEASES

ANNUAL REPORT

October 1, 1979 through September 30, 1980

I. MISSION

The broad program goals of the Division of Lung Diseases are more effective diagnosis, treatment and, ultimately, prevention of pulmonary disease. To these ends, the Division emphasizes seven program areas: structure and function of the lung (includes lung development); chronic obstructive lung diseases (emphysema, chronic bronchitis, asthma); pediatric pulmonary diseases (neonatal respiratory distress syndrome, cystic fibrosis, bronchiolitis); fibrotic and immunologic interstitial lung diseases; pulmonary vascular diseases (pulmonary hypertension, pulmonary edema); respiratory failure; and prevention, control and education. These programs are implemented through four branches: Structure and Function; Airways Diseases; Interstitial Lung Diseases; Prevention, Education and Manpower.

In addition to supporting investigator-initiated regular research, program project and training grants, research career awards, and young investigator research grants, the Division has stimulated research or training in specific areas through goal-oriented initiatives. Grants for specialized centers of research (SCORs) support interdisciplinary investigations with a clinical focus. A grant for a lung research and demonstration center supports demonstration and education projects to prevent and control pulmonary diseases. To meet the need for additional research and clinical manpower, the Division initiated the Pulmonary Academic Award and Pulmonary Faculty Training programs. Research contracts are used to foster targeted pulmonary research and development projects.

II. PROGRESS TOWARD OBJECTIVES

The investigator-initiated research grant continues to be the major source of innovative fundamental research. It contributes not only to our understanding of the normal lung and the abberant processes involved in respiratory disorders, but also leads to improvements in the diagnosis and management of pulmonary diseases. From time to time the Division supplements these programs by issuing requests for applications (RFAs) or for contract proposals (RFPs) to encourage exploration of problems not being addressed, or being insufficiently addressed, by investigator-initiated grants. The areas where stimulation is needed are identified through working groups, workshops and task forces, as well as by the Pulmonary Diseases Advisory Committee and the Division's professional staff. One program area in the Division's National Plan continues to depend almost entirely on special initiatives; namely, the Prevention and Control program.

A working group on the *Natural History of the PiZZ Subject* brought together investigators who had collected pulmonary function data on more than 240 subjects identified as homozygous for alpha-1-antitrypsin deficiency (PiZZ phenotype), a condition found in only about one in 1,500 individuals. They discussed their findings, and are now summarizing the data on each subject in uniform format. At a second meeting, these retrospective data will be analyzed and the working group will consider the feasibility of a trial on alpha-1-antitrypsin replacement therapy for individuals who are prone to emphysema because they are deficient in this antiprotease. Another working group was convened to discuss *Terminology of Chronic Obstructive Lung Diseases*, because diversity in diagnostic criteria and in usage of terminology have been obstacles to collection of reliable data on incidence, prevalence and morbidity of these diseases. This is a troublesome problem identified by the Task Force on Epidemiology of Respiratory Diseases as hampering epidemiologic research.

The Division also sponsored five workshops to enable experts to discuss the current state of knowledge in the field and recommend new directions to fill gaps in current research programs. Reports of these workshops, which are distributed to the biomedical community, serve as a stimulus to new investigative approaches as well as providing a source of ideas for special initiatives of the Division of Lung Diseases. A workshop on *Mechanics of Lung Parenchyma* discussed what is known about the biochemical and morphologic characteristics of lung connective tissue components and the relationship between the chemical structure and mechanical features of lung parenchyma. The *Scientific Basis of In-Hospital Therapy* was the subject of a workshop that examined data on therapies currently used to manage hospitalized patients who develop respiratory complications, and identified the kinds of additional information required for an assessment of the efficacy of different modes of therapy. A "hands-on" experience for investigators wishing to use lung morphometry in their research was provided at a 3-day workshop on *Quantitative Morphology of the Lung* for a limited number of qualified researchers. A workshop on *Noninvasive Methods to Monitor Intracellular Events* brought together pulmonary researchers, and biochemists and biophysicists who have developed techniques that might be able to be used to monitor intracellular changes associated with abnormal levels of oxygen, or acid-base balance disturbances. The participants explored the feasibility of applying such techniques to the noninvasive assessment of blood gas abnormalities in patients with respiratory disturbances. The Division of Lung Diseases and the Institute of Allergy and Infectious Diseases jointly sponsored a workshop on *Basic and Clinical Aspects of Granulomatous Diseases*. Participants included basic scientists, especially immunologists, as well as investigators concerned with clinical problems. It was designed to bridge the gap between what is known about basic inflammatory processes and the clinical aspects of sarcoidosis and other granulomatous diseases.

The *Task Force on Epidemiology of Respiratory Diseases* completed its deliberations this fiscal year and its report has been distributed to the biomedical community. The report emphasizes the extent to which respiratory diseases are underreported, resulting in a misleading picture of the true magnitude of health problems associated with these disorders. The report identifies the kinds of basic and clinical research, technology development, and innovative approaches to statistical methodology that are necessary before certain types of epidemiologic studies can be effectively pursued. It also makes specific recommendations for an epidemiology program that will improve our knowledge of the occurrence of the various respiratory diseases, exogenous risk factors, especially in working environments, host factors that predispose an individual to respiratory disease, the long-term consequences of respiratory disorders of infancy and childhood, and the natural history of chronic respiratory diseases.

A *Task Force on Pulmonary Technology*, which held its first meeting in January 1980, agreed to work through five task groups that include additional experts. They have met and are now preparing chapters on respiratory failure and obstructive lung diseases; fetal, neonatal and pediatric medicine; noninvasive lung imaging; pulmonary vascular and interstitial lung diseases; and technology development, evaluation and standardization.

As a special initiative to encourage animal studies of the effects of nutrition on the lung, which has been little studied compared with the effects of other organ systems, the Division issued an RFA for *Studies of Nutritional Status and Nonrespiratory Lung Function*. Such fundamental investigations are needed for better insights into the role of nutrition in the etiology, pathogenesis and management of diseases of the lung.

Two other special initiatives are an outcome of the recommendations of the Task Force on Prevention, Control and Education in Respiratory Diseases. One was an RFP on *Identification of Variables Associated with Maintenance of Nonsmoking in Ex-Smokers*. It invited studies addressed to the problem of recidivism that is common to all smoking cessation programs. Groups with experience in research on smoking cessation and maintenance of nonsmoking behavior were encouraged to submit proposals for obtaining additional data on variables that seem to be predictive of successful long-term maintenance of nonsmoking. The other RFP invited development of the *Methodology for Evaluation of Primary Physicians' Utilization of Knowledge of Respiratory Diseases*. It is well known that there is a disparity between what a physician knows about a disease and what use is made of this knowledge in his or her daily practice. There is a need for an evaluative methodology that can be used to assess the extent to which physicians are using their knowledge and skills in their community practice relative to diagnosis and management of respiratory diseases.

The Division of Lung Diseases is committed to periodic assessment of its special programs. Because 14 awardees supported through the Pulmonary Academic Award Program have now completed the 5-year term of this award, the Division issued an RFP to obtain an *Evaluation of the Pulmonary Academic Award Program*. Respondents were asked to present proposals for comparing these 14 awardee schools, which had minimal pulmonary programs prior to the award, with a group of similar schools that had not received a Pulmonary Academic Award.

The Pulmonary SCOR (specialized centers of research) program is now open to national competition for centers addressed to one of four disease categories: chronic obstructive lung diseases, pediatric pulmonary diseases, fibrotic and immunologic interstitial lung diseases, and pulmonary vascular diseases. Applications are due September 1980 for award in fiscal 1982 when the project periods end for current SCOR grants in these categories. SCORs addressed to respiratory failure are not included in this competition because their project period does not end until fiscal 1983.

Of considerable interest are new research emphases emerging in several areas of investigation, sometimes as a result of technologic advances, in other instances because changes in the environmental or industrial scene have introduced new challenges to researchers. Some recent trends are identified below and specific examples given in the next section of this report.

Technical improvements in the management of infants with neonatal respiratory distress syndrome have dramatically improved survival rates. But it is now evident that surviving infants are at risk of a chronic pulmonary disorder--bronchopulmonary dysplasia. A recently issued report of a workshop on this disorder identifies the urgent need for better diagnostic criteria, studies of the natural history of the disease, and clinical and laboratory research to identify basic mechanisms involved in its etiology and pathogenesis. All of these lines of investigation are necessary to more effective management and the prevention of bronchopulmonary dysplasia, which appears to be a mounting problem as more premature infants survive. Another disease of childhood in which improved management has prolonged life is cystic fibrosis. As children with this disease are surviving into adulthood, the problems of management are now similar to those for other chronic obstructive diseases of the lung, such as emphysema and chronic bronchitis. Unfortunately, treatment for these disorders is at present only palliative, but still cannot reverse the deterioration of lung function.

Physiologic studies under conditions of increased pressure are of rapidly increasing interest because technical advances have more than doubled the depth at which men can work in the ocean, and because exploration for oil, retrieval of sunken treasures, and even deep-diving as a recreational sport, are exposing more and more individuals to hyperbaric conditions.

Investigators are becoming more aware of exposures to hazardous agents that affect the lung not only in the workplace, but in the general environment in the vicinity of industries, or in communities where the population is exposed to noxious agents as a result of accidents such as freight train wrecks. Epidemiologists and other types of researchers are giving greater attention to such events and undertaking follow-up studies of exposed populations or individuals.

Another important recent trend is the growing awareness of the importance of the social and psychological consequences of pulmonary disease. This is evident in the greater emphasis on "quality of life" in assessing the efficacy of treatment in various clinical trials.

Although basic studies of the effects of cigarette smoking have revealed important information about its role in altering specific lung cells and enzyme systems, other aspects of the pervasive problem of smoking are still elusive. The effects of cigarette smoking on nonsmokers, a question with implications for public policy, is being extensively studied. But few hard data are available to confirm the widely held impression that nonsmokers are harmed by cigarette smoke in the environment. Interest in behavioral studies is also increasing because investigators are challenged by the baffling fact that many persons successfully stop smoking on their own, while others, who turn to smoking cessation clinics for help, have short-term success but usually return to the smoking habit.

III. HIGHLIGHTS OF RECENT SCIENTIFIC ADVANCES

This section identifies some important advances reported by investigators within the past 2 years. Because of the dimensions of the Division's research program and the breadth of emphasis of the investigations it supports, this summary fails to do justice to the many investigators whose work cannot be represented in this brief account. Some of their accomplishments have been covered in prior reports, others will be presented in the future.

Risk Factors

Several findings of considerable interest have emerged from studies of host factors that predispose individuals to respiratory disorders, and of environmental factors that increase risk of such disorders in exposed persons. A challenging question is why some persons exposed to environmental hazards develop respiratory disease, while others, similarly exposed, remain free of disease.

Among host factors believed to modify susceptibility are differences in immunologic characteristics of individuals. A striking example of individual differences has been found in pigeon breeders. All those with pigeon breeder's disease (a form of hypersensitivity pneumonitis) and about half of those free of this disease had serum antibodies against the antigens from pigeon droppings. Hence, the serum antibodies indicate exposure but not presence of disease. It was, however, possible to discriminate between sick and well breeders through studies of cellular immunity in lymphocytes obtained by lung lavage. Lung lymphocytes of sick breeders responded to pigeon antigen whereas lymphocytes of well breeders did not, indicating differecnes in cellular immunity in these two types of individual. It is also of interest that there are individual differences in the course of the disease, some sick breeders maintaining a stable course despite continued exposure to the antigen, while others show progressive deterioration. It has now been shown that initial tests of pulmonary function can be used to predict the course of the disease. Those with abnormal pulmonary function on initial tests tend to deteriorate over the years, whereas those with normal pulmonary function are likely to maintain a stable course even when exposure to pigeon antigen continues.

In the past few years a variety of studies of individual differences in ventilatory responses to physiologic stresses have revealed a familial tendency in these responses that affects the individual's capacity to resist, or cope with, respiratory disease. Among recent observations, of particular interest is evidence for a familial tendency to defective ventilatory responsiveness that appears to be a predisposing factor for sudden infant death syndrome. Previous comparisons of identical and nonidentical twins, of offspring of hypoventilating and nonhypoventilating patients with chronic obstructive lung disease, and of nonathletic parents and siblings of successful endurance athletes who have decreased ventilatory drive, all point to the existence of a familial determinant of diminished ventilatory drive. In a recent study, the ventilatory responses of a group of parents whose children were victims of sudden infant death syndrome were compared with the responses of a group of parents with no family history of this syndrome. On the basis of two procedures that increase ventilatory responses in normal subjects, the investigators found significantly lower ventilatory responses in the group of parents of infants who died of sudden infant death syndrome than in the control group. These observations suggest that persons who manifest low ventilatory responses to carbon dioxide and to increased airway resistance (the two test procedures used in the study) may be at risk of having children susceptible to sudden infant death syndrome.

Several recent studies point to a relationship between individual differences in reactivity of the airways and a number of lung disorders. In an epidemiologic study to determine the relative importance of different risk factors for development of airways obstruction, hyperreactivity of the airways (determined by methacholine challenge) was found to be more frequent in sons of patients with chronic obstructive pulmonary disease than in sons of a control group without this disease. These observations suggest that a familial component of airway obstruction is related to airway hypersensitivity, and that a test of reactivity may provide a sensitive index of risk for developing chronic obstructive disease of the lung. These findings are consistent with observations from a natural history study of airways obstruction in nonsmokers in a rural population minimally exposed to air pollution. In this population, a history of asthma (which is typified by airway hyperreactivity) was significantly associated with progressive ventilatory deterioration and with mortality. An association has also been found between a family history of asthma and development of bronchopulmonary dysplasia in a group of 39 infants who survived neonatal respiratory distress syndrome. Of the 17 infants who developed bronchopulmonary dysplasia, 75 percent had family members with a history of physician-diagnosed asthma, and 59 percent had family members who had been hospitalized for asthma because of its severity. In contrast, among the 22 infants who survived neonatal respiratory distress syndrome but did not develop bronchopulmonary dysplasia, only 33 percent had family members with diagnosed asthma, and only 14 percent had family members who had been hospitalized because of it.

Despite vigorous public debate about policies to shield nonsmokers from exposure to cigarette smoke, there are few hard data to support the view that "passive smokers" are at increased risk of emphysema and chronic bronchitis. A recent study provides the first quantified evidence that nonsmokers exposed for 20 or more years to cigarette smoke in their workplace have a small, but statistically significant, diminution in function of the small airways. This evidence of a physiologic change does not, however, answer the question of whether such individuals are at greater than average risk of chronic diseases of the lung. Another study addressed to this issue examined the children of smoking parents. An effect on lower levels of pulmonary function and on the symptom of wheezing in children 5 to 10 years of age was significantly associated with level of parental smoking. The association with wheezing was present in children who had never had a diagnosis of asthma as well as in those with diagnosed asthma.

Diagnosis

A variety of approaches are being addressed to development of (1) more reliable as well as more sensitive measures of pulmonary function, (2) diagnostic techniques that are suitable for children who are too young and for patients who are too ill to cooperate in use of standard diagnostic procedures, and (3) tests that can be used repeatedly to monitor the course of a disease and progress during therapy. Some innovations are modifications of widely used methods, others have drawn on recent advances in knowledge of the biochemical and immunologic alterations associated with specific diseases.

Among noteworthy advances in development of noninvasive techniques for assessing lung function is a canopy that incorporates a spirometer and is combined with a computer system. It has the advantage of eliminating the need for the mouthpiece that is used in other types of spirometry, and can be used to monitor changes in breathing volumes and flow rates in severely ill patients during the course of therapy. Another noninvasive approach uses full-chest fluoroscopy that permits examination of patients while maneuvers associated with pulmonary function tests are carried out. Changes in regional volumes at various lung volumes and in various positions are now being studied.

Because compliance (extensibility) of the lung diminishes in chronic obstructive and fibrotic lung diseases, techniques that measure early changes in this mechanical property are of considerable importance, especially if they can be performed noninvasively. Particularly promising are methods that introduce a sound wave into the airway and measure its transmission or reflection in the tracheobronchial tree. Improvements in this technique may make it possible to distinguish between changes in large and small airways, and a recent advance that uses a random noise, instead of a wave of a single frequency, can provide data in a few seconds. These techniques are of special value in studies of small children, and should be applicable in epidemiologic surveys, or to screen population groups for disease.

Several research groups are refining transcutaneous methods that permit continuous assessment of arterial oxygen and carbon dioxide pressures without the need for arterial catheters or repeated withdrawal of blood samples. Although skin sensors are commerically available, they have shortcomings that are being corrected in studies of new probes and sensor designs.

Biochemical and immunologic markers are a valuable clue to alterations associated with specific diseases and have the advantage of being identifiable in small samples of serum, urine, or lung lavage fluid. A sensitive spectrophotometric assay has demonstrated an increase in levels of angiotensin-converting enzyme in the serum of patients with sarcoidosis, a granulomatous disease that is difficult to diagnose in its early phases. The observation that enzyme levels appear to correlate with severity of disease, and to decrease in patients undergoing conventional therapy, suggests its potential value in monitoring the clinical course in patients with sarcoidosis. Another enzyme of potential diagnostic value is collagenase, which digests the structural protein collagen. It is found in lung lavage fluid from patients with idiopathic pulmonary fibrosis but not in lung fluids of normal subjects. The presence of collagenase suggests that collagen degradation, followed by disordered synthesis, may be important in the pathogenesis of this type of fibrosis, which is still of unknown etiology. The enzyme collagenase may also prove useful in monitoring the progression of the disease process.

A radioimmunoassay for desmosine and isodesmosine offers a promising approach to early detection of elastin breakdown in emphysema. These peptide fragments, which are unique as crosslinks in the elastin molecule, are being intensively investigated in animals and humans to quantify their levels in plasma and urine. These studies are a first step to development of what may be an inexpensive, noninvasive way to diagnosing emphysema in its early stages.

A simple physical test for assessing the viscosity of amniotic fluid has been shown to predict reliably whether the neonate is likely to develop respiratory distress syndrome. The procedure is simple enough to be performed at the bedside by the attending physician or even by nonprofessional personnel, requires only about 20 drops of amniotic fluid and is a great improvement over assessment of the widely used L/S (lecithin/sphingomyelin) ratio.

Therapy

The problems of treating and managing patients with respiratory diseases are of such complexity that definitive solutions are difficult to achieve. Some of the problems are being addressed through clinical trials, others are being explored through fundamental studies of agents of potential value in therapy. Under examination are confounding factors, such as individual constitutional and psychologic differences, that influence the outcome of a given mode of therapy. Neurophysiologic and psychological assessments are also being used to determine how different modes of therapy affect the patient's life.

Current clinical trials of intermittent positive pressure breathing (IPPB) and nocturnal oxygen therapy for chronic obstructive lung disease, and of antenatal steroid therapy for prevention of neonatal respiratory distress syndrome, are on schedule but the data are still blinded.

With the long-term goal of developing a replacement therapy for individuals deficient in alpha-1-antitrypsin, researchers are addressing various aspects of this problem. Elastase inhibitors with a longer half-life than naturally occurring compounds have been synthesized and shown, in animal studies, to reduce the severity of experimentally-induced emphysema. A recently developed carrier system of albumin microspheres delivers these inhibitors directly to the lung and has been found to increase the effectiveness and decrease the side effects of the inhibitors in animals.

A 3-year exploratory study of normal male subjects, to determine whether the movement of gas in and out of the lung can be improved through exercise training of ventilatory muscles, has given encouraging results and is now being extended to a group of patients with chronic obstructive lung disease. Moreover, a simple, inexpensive device has been built that allows the patient to continue the ventilatory muscle training at home, after completion of the testing program. These patients will be followed for several years to determine the efficacy of the home treatment.

A study in progress for some years has now advanced to the point of developing a "battery of asthma illness behavior" that enables investigators to identify personal styles or behavior patterns that can defeat medical regimens. The battery provides the basis for predicting whether a given patient is likely to use anti-asthma medications in ways that are potentially dangerous to himself, misleading to his physician, and a threat to his medical management.

There is mounting evidence that adequate nutrition is essential to the maintenance of normal function of muscles of the diaphragm, ribs and chest wall, all involved in normal breathing. It has recently been reported that inadequate nutrition in patients with respiratory disease compromises their ability to cope with the disorder. Moreover, in patients with no disease of the lung or chest wall, but who have severe nutritional disturbances from disease elsewhere in the body, performance in routine breathing tests is reduced and the strength and endurance of their respiratory muscles is about one-half normal. These are important findings because, when sufficiently severe, breathing impairment may result in acute respiratory failure. Another study points to the complexity of the problem of achieving optimal nutritional, as carbohydrate loading in patients with compromised pulmonary function was found to cause an increase in carbon dioxide levels that can precipitate respiratory distress.

IV. REPORTS OF WORKSHOPS, MEETINGS, OTHER

A. Workshop Reports

- Report of Workshop on Bronchopulmonary Dysplasia
  December 4-6, 1978
  NIH Publication No. 80-1660

- Report of Workshop on Mechanics of Lung Parenchyma
  September 23-25, 1979
  NIH Publication No. 80-2109

B. Evaluative Reports

- Evaluative Report on Completed Contracts Awarded in Response to RFP NHLI-75-5: Identification of Lung Cells
  NIH Publication No. 79-1664

- Evaluative Report on Completed Contracts Awarded in Response to RFP NHLI-74-7: Development of an In Vitro Diagnostic Test for Sarcoidosis
  NIH Publication No. 79-1668

C. Other Reports

- Excerpts from Pulmonary SCOR Progress Reports (5 Volumes)
  October 1, 1979 (Limited distribution)
- Report to National Heart, Lung, and Blood Advisory Council
  November 29-30, 1979 (Limited distribution)
- Report of Lung Cell and Organ Culture Course: W. Alton Jones Cell Science Center
  November 13-16, 1979 (Limited distribution)
- Extracorporeal Support for Respiratory Insufficiency: A Collaborative Study in Response to RFP NHLI-73-20
  December 1979 (Limited distribution)
- National Heart, Lung, and Blood Institute, Division of Lung Diseases Program Report: Fiscal 1979
  NIH Publication No. 79-1659

V. MAJOR PROBLEM AREAS

The problems identified below are the same as those reported last year. The situation has become even more critical in the interim since the 1979 report.

A. Funds for a Prevention and Control Program

A problem that is not new, but has assumed greater importance since the report of the Task Force on Prevention, Control, and Education in Respiratory Diseases has been issued, is the absence of funds specifically appropriated for a program that was authorized in the Act of 1972.

To the greatest extent possible, the Division of Lung Diseases has drawn upon funds for research to develop a prevention and control program. However, any further development would compromise the research grant program, unless additional funds are made available for the prevention and control effort.

B. Funds for New Initiatives

Because the Division is committed to investigator-initiated research as its first priority, and because the number of research grant applications addressed to pulmonary diseases has markedly increased within the past two years, it is now very difficult to assign funds for activities that are necessary to fill gaps in research areas that still need attention and that are important to national health.

Division of Blood
Dis. & Resources

ANNUAL REPORT

DIVISION OF BLOOD DISEASES AND RESOURCES
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1979 Through September 30, 1980

The Division of Blood Diseases and Resources seeks to improve the prevention, diagnosis, and treatment of blood diseases and related disorders and to assure the appropriate, efficient, and safe use of an adequate supply of high quality blood and blood products. Its programs deal with four major areas: (1) Thrombosis and Hemostasis, (2) Red Blood Cell Disorders, (3) Sickle Cell Disease, and (4) Blood Resources. Each program encompasses fundamental and clinical research; targeted applied research; professional development and training; and prevention, education, and control activities. The aim at all times is the rapid, but orderly, development and application of new knowledge so as to reduce morbidity, mortality, and the costs of health care. To best use its scarce resources, the Division actively coordinates its efforts with those of other federal and nonfederal agencies and organizations. In developing and supporting its programs, the Division of Blood Diseases and Resources seeks to use all appropriate support mechanisms including: investigator-initiated research projects; program project grants; goal-oriented centers such as Specialized Centers of Research (SCORs), and a National Research and Demonstration Center; targeted research and development contracts; and evaluation and technology transfer projects. The long-range objectives of each Division program and highlights of fiscal year 1980 progress toward the attainment of these objectives are summarized in the following paragraphs.

THROMBOSIS AND HEMOSTASIS

The long-range objective of this program is to develop new knowledge which could be applied to prevention, diagnosis, and treatment of thrombosis and hemorrhage. The program has three subprograms. (1) Thromboembolic Disorders: This subprogram covers both arterial and venous thrombosis. Although the problems posed by these two disorders differ in many ways, elucidation of hemostatic mechanisms, including the roles of soluble clotting factors and their inhibitors as well as platelets and their secreted products, is important to both. The ultimate goal is the reduction of mortality and morbidity from venous thrombosis and occlusive arterial thrombi. (2) Hemorrhagic Disorders: This subprogram supports studies involving all aspects of the inherited bleeding disorders and also encourages basic research involving acquired disorders of coagulation. The overall goal is to develop a better understanding of the genetic and pathologic mechanisms underlying hemophilia and other bleeding disorders so as to develop improved diagnostic techniques and more effective specific treatments. (3) Platelet Disorders: This subprogram sponsors studies involving platelet function in relation to platelet disorders. The goals are to develop new knowledge of functional roles of platelets in the human biological defense mechanisms and to apply this knowledge to the development of more effective therapies for individuals suffering from congenital and acquired platelet disorders.

Highlights

Some hemophiliacs develop inhibitors which block the effects of the antihemophilic factor VIII. Although factor VIII in large amounts and a blood component called prothrombin complex concentrate (PCC) both exert a therapeutic effect and neither is completely satisfactory. A just-completed study shows that many of these patients experience reduced pain and less bleeding into joints when given PPC rather than factor VIII.

An intricate balance is required among the numerous factors contributing to coagulation. A recent study demonstrated that the measurement of the action of two factors, thrombin and plasmin, in plasma may help identify patients with an imbalance which increases their risk of thrombosis.

Antithrombin III (ATIII) is a glycoprotein which inhibits thrombin and other proteolytic enzymes involved in blood coagulation, fibrinolysis, and in the plasma kallirein and complement systems. Basic research has elucidated much about the structure, function, and processes of this protein including the discovery that antithrombin III represents a new family of plasma inhibitor structures. In vivo, much as been learned about how ATIII regulates thrombin. Stemming from this new knowledge is the hypothesis that ATIII may act in the early steps of the coagulation cascade and that this action may be greatly magnified by the endpoints of clot formation and degradation.

RED BLOOD CELL DISORDERS (EXCLUSIVE OF SICKLE CELL ANEMIA)

This program is concerned with the development of new knowledge in the areas of Cooley's anemia and aplastic and refractory anemias, as well as hemolytic anemias. It is composed of three subprograms. (1) Cooley's Anemia and Other Hemoglobin Disorders: The long-range goals are to improve patient treatment and extend the life span of afflicted individuals, as well as to improve the quality of life of victims of these disorders; to further elucidate basic defects, both genetic and biochemical, as a basis for developing techniques which can cure or prevent disease; to develop an understanding of the basic pathogenic mechanisms underlying the clinical aspects of the hemoglobinopathies; and to expand our understanding of the mechanisms of control of oxygen transport. (2) Erythropoiesis: The long-range objectives are to apply the knowledge gained from studies on erythropoiesis to the cure of stem cell disorders, such as aplastic anemia, and other disorders of red blood cell production; and to obtain adequate supplies of erythropoietin for research and clinical use. (3) Red Blood Cell Membrane Enzyme Systems: The long-range goals are to further elucidate red cell membrane structure and function as well as gain additional insight into the process of intracellular metabolism; and to use this information to improve the health of patients afflicted with various hemolytic anemias.

Highlights

Patients with Cooley's anemia are treated with frequent transfusions. As a result, excess iron collects in various organs. Desferrioxamine (DF) is an iron chelating agent used to remove this excess iron. Currently, the mode of administration of the DF is subcutaneous infusion that takes several hours. Recent study results suggest that a more effective mode of administration may be packing the DF in the ghosts (membrane shells) of red cells. Data show that one unit of DF in red cells removes nine times as much iron as DF administered by current methods.

Iron deficiency, and the resulting anemia, affects between 10 and 20 percent of the world's population. A procedure combining assays for hemoglobin, transferrin saturation, free erythrocyte protoporphyrin, and serum ferritin has been developed to evaluate a population in regard to concentrations of iron in the blood and to the causes of such amounts. This procedure may be particularly useful for evaluating the effectiveness of iron supplementation and fortification programs.

SICKLE CELL DISEASE

The mission of this program is to reduce morbidity and mortality caused by sickle cell disease. The long-range objectives are to continue basic research into the pathophysiology of the disease process at the molecular, cellular, and clinical levels; to develop improved methods of clinical care; to develop a more rational approach to patient management based on the latest scientific advances; to provide accurate, up-to-date information to health care providers and consumers; and to evaluate the effectiveness of education, screening, and counseling programs.

Highlights

Advances in the use of specific restriction endonucleases to dissect, with precision, the nucleic acid sequences of genes are rapidly leading toward the development of techniques for the prenatal diagnosis of not only sickle cell disease but also other hereditary hemoglobinopathies such as Cooley's anemia. Because amniotic fluid samples rather than fetal blood samples are used, the safety of these diagnostic procedures is greatly improved.

The abnormal shape and rigidity of the sickled erythrocyte have generally been held responsible for the microvascular occlusions of sickle cell disease. Because there is no correlation between the clinical severity of this disease and the presence of sickled erythrocytes, it has long been suspected that other factors must be involved in regulating the interaction between cells or between cells and capillary walls. Recently, investigators have found that sickle cells adhere to the vascular endothelium much more strongly than normal erythrocytes. This adherence may be a pathogenetic factor in the microvascular occlusions characteristic of sickle cell disease. This raises the possibility that vasocclusion may be controlled not only by interfering with sickling, but by some mechanism which renders the cells less "sticky."

BLOOD RESOURCES

The mission in blood resources is directly related to the National Blood Policy goal of an adequate supply of high quality blood and blood products. In pursuit of this goal, the blood resources program supports activities to improve donor and recipient safety, blood component therapy, transplantation biology, blood substitutes, and all aspects of the management of blood resources including collection, fractionation, preservation, and distribution. This program consists of five subprograms with the following objectives. (1) Blood Resources Management: Foster the efficient use and assure an adequate supply of high quality blood and blood products; promote more effective planning in the management of the national blood resource through collection and analysis of national blood resource data; and encourage improved blood resource sharing both regionally and nationally. (2) Blood Safety: Promote basic investigations centering on immunohematologic problems particularly concerning blood group antigens and antibodies, the RH complex, and red cell antibody and complement interactions; promote studies to eliminate hepatitis as a transfusion-transmitted disease; and promote studies that will lead to greater safety for donors of blood and blood components. (3) Blood Substitutes: Pursue further development of newly synthesized fluorocarbon compounds for use as blood substitutes in transfusion therapy, organ perfusion, and other promising applications. (4) Blood Component Therapy: Develop definitive guidelines for the clinical use of blood components including packed cells, albumin, granulocytes, and platelet concentrates; explore use of buffy coat as a source of human interferon; and develop new methods for plasma fractionation, including preparation of chemically useful trace components. (5) Transplantation Biology: Support fundamental research in immunology, immunogenetics, and other aspects of transplantation biology.

Highlights

Post-transfusion hepatitis develops in approximately seven to ten percent of blood recipients or between 200,000 and 300,000 persons annually. Ninety percent of these cases are non-A,non-B (NANB) hepatitis for which the causative agent(s) are unknown. However, a collaborative study has shown that blood containing elevated levels of the enzyme alanine aminotransferase (ALT) causes a high risk of infection for NANB hepatitis. Therefore, screening blood samples for ALT may be useful as an interim assay for reducing the incidence of post-transfusion hepatitis.

The first activated-carbon hemoperfusion device designed specifically for rescue and life support of young children suffering from ingestion of poison(s) has been developed and is being tested in vivo and in vitro. The availability of this device could help reduce the annual toll of children's lives and health caused by ingestion of household cleaners, pesticides, and drugs.

PROFESSIONAL TRAINING AND DEVELOPMENT

As a focus of blood research training, the Institute cooperates with other NIH Institutes, professional societies, and other Governmental agencies to carry out a continuing assessment of the national personnel needs for research in blood diseases and blood resources. To assist in meeting these needs, the Institute currently supports 188 trainees in blood diseases and resources.

Division of Extramural Affairs

NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
DIVISION OF EXTRAMURAL AFFAIRS
Annual Report
October 1, 1979 - September 30, 1980

The Division of Extramural Affairs is responsible for formulating, coordinating and evaluating Institute-wide policies and procedures for research contracts, grants, and training programs. The Division is the focal point for providing advice to the Director, NHLBI, on research contract, grant, and training program policy and procedure. It also represents the Institute on overall NIH extramural grant and collaborative program policy committees, coordinates such policies within NHLBI, and coordinates all of the Institute's research and training program activities with the National Heart, Lung, and Blood Advisory Council. Other major responsibilities of the Division include: (a) grant and contract management and processing services for the Institute, (b) initital scientific and technical merit review of certain competing grants and all research contracts for the Institute, and (c) committee management functions for the entire Institute.

The Division continues to serve as the primary liaison to the National Heart, Lung, and Blood Advisory Council, and has continued to develop procedures and mechanisms to facilitate the Council's review of programs and applications and the review, approval, and implementation of new initiatives and program plans. The Council meets at least four times a year. Several Council working groups have been established and these continue to contribute significantly to the operation of the Institute. Some of these working groups emphasize application review while others emphasize program review and development.

During FY 1980 the Division continued to provide a broad range of services for the entire Institute. These included:

1. Initial scientific and technical merit review of competing research grant, contract, and training proposals.

2. Management functions for research grants, contracts, and training/manpower awards.

3. Maintenance of official files for all grant and contract programs, including regular updating to keep them current.

4. Obtaining all final reports and accomplishing the close out of terminated grants and contracts.

5. Preparation of review materials for Council, staff, and Institute initial review groups.

6. Preparation of official and summary minutes of Council meetings and summary statements of Special Council actions.

7. Operation of the Program Policy and Procedures Office.

8. Committee management functions.

The initial scientific and technical merit review of special research grant applications, training grant applications, and research and development contract proposals has continued to be the main focus of Review Branch activities. In FY 1980, the types of reviews in the grant program included:

Pulmonary Academic Awards

Preventive Cardiology Academic Awards

National Research Service Awards for Institutional Training Grants

Special Emphasis Research Career Award (SERCA) in Diabetes

Minority Hypertension Research Development Summer Program

Program Project Grants

Comprehensive Sickle Cell Center Supplemental Grants

Specialized Center of Research (SCOR) Grants: Hypertension

Specialized Center of Research (SCOR) Grants: Thrombosis

National Research and Demonstration Center Program (Supplement)

Cooperative Clinical Trial Research Grants

Research Demonstration and Dissemination Project Grants

Education Project Grants

Conference Grants

Research Project Grants

Continuing Education Training Program

Clinical Investigator Awards

Studies on the Role of the Microcirculation in Hypertension (RFA)

Nutritional Status and Nonrespiratory Lung Function (RFA)

In addition, numerous new, renewal, and unsolicited contract proposals were reviewed.

The Review Processing Section continued to perform its diverse functions in a satisfactory manner in spite of the threat of large scale down-gradings and personnel changes. This threat still lingers and has had a serious effect on the morale of the grants clerks and grants technical assistants in this section.

The Grants Operations Branch was involved in the Institute's grant activities from the pre-application (planning) stage to the close-out of terminated grant projects. Branch staff are often contacted by grantee institution staff concerning the best method of presenting consortium budgets, requests for research patient care costs, alterations and renovations, etc. The Branch was also actively involved in site visits, Council preparation, post Council - pre-award budget negotiations, issuing award notices (approximately 3,200 totaling in excess of $347,000,000 for all the Institute programs) as well as the post award fiscal and administrative management of those awards. The Branch served as an important interdivisional coordinator, and senior representatives of the Branch continued to be effective in assisting NIH-OD staff in the development and analysis of NIH grant policy and procedure. The Branch's continued efforts to improve management practices and procedures have undoubtedly resulted in considerable dollar savings for the Institute.

The Processing Section of the Grants Operations Branch continued to improve the maintenance of grant files (pending, active, terminated) and continues to be responsible for the preparation of all grant encumbrance lists. The Section also received, reviewed, and filed or appropriately forwarded items pertaining to NHLBI grants and awards (e.g., activation notices, appointment forms, and day-to-day correspondence). The Section was also responsible for the preparation of Council Books and for the duplication of some summary statements and supplemental material. The Section also provided information, duplicated from the files, to the Institute's Privacy Act and Freedom of Information Coordinators.

The Contracts Operations Branch, responsible for the administrative and business management aspects of the NHLBI contract program, continues to be involved in a variety of activities: presolicitation, solicitation, evaluation of proposals, determination of competitive range, negotiation, award, post award administration, termination and closeout of completed contracts awarded by the Institute.

During FY 1980, Secretary Harris has continued a major survey of the DHHS grants and contracts program, and ordered a number of significant moves to correct deficiencies in these programs. As a result, fewer noncompetitive contracts are awarded by DHHS components and a major shift in the distribution of award dates will occur. To more evenly distribute workload throughout the fiscal year, an effort has been mounted to reduce the number of awards in the fourth quarter of the fiscal year. New restrictions have been levied upon awarding components limiting them to awarding no more than 30% of contracts during the fiscal year's fourth quarter. No more than 12% of the total may be awarded during each of the last three months of the fiscal year.

In conjunction with Secretary Harris' initiatives, a program has continued to certify contracting officers and contract specialists. To date, eleven NHLBI contract specialists have been certified. The contracts program was comprised of approximately 345 contracts and reimbursable agreements, representing approximately $73,000,000 during FY 1980. The Branch staff continued to work closely and effectively with the program staff but with the appropriate and necessary independence that denotes professionalism.

Mr. George Campion who has served as Section Chief and Contracting Officer in the Division of Lung Diseases Contracts Section, has retired. Mr. Douglas Frye has been chosen as his successor in this major NHLBI research contract program.

In January 1980, Dr. W. Glen Moss retired as the Deputy Director of the Division. This essential position has remained vacant since his departure, and we have still not received permission to recruit an SES replacement. This has placed an extremely heavy burden on the Division Director; the Division is still awaiting the go-ahead to begin the steps necessary in filling this vital position.

The staff has continued to perform in excellent fashion, but morale has suffered somewhat both from the threat of down-gradings to be carried out in the GS-301 series, and from down-gradings that have actually occurred. It is hoped that the NIH will be successful in ending the threat of these down-gradings in the GS-301 series.

Overall, the Division has space to perform its various missions although the space in the Westwood Building is still spread over four floors and this causes considerable inconvenience. There are two specific areas where space needs are becoming very acute, however. In the Processing Section of the Grants Operations Branch, the workload relating to Council preparation has continued to grow so that the space allotted for that purpose is now quite cramped. In the Contracts Operations Branch, the accommodations for staff assigned to the Federal Building leave much to be desired.

Staff turnover has exceeded the expected level, but we have managed to fill most vacancies. Additional positions for understaffed areas, especially processing units, are needed to insure maximum efficiency and productivity.

Division of Intramural Research

INTRAMURAL RESEARCH

THE NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

ANNUAL REPORT

October 1, 1979 - September 30, 1980

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Annual Report
Section on Enzymes
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1980 - September 30, 1981

A. Mechanism of Enzyme Action

(a) Glutamine Synthetase. In earlier studies, the catalytic cycle of the biosynthetic reaction catalyzed by the Mg(II)-dependent unadenylylated form of glutamine synthetase (GS) was elucidated by monitoring the intrinsic protein fluorescence changes accompanying substrate binding and the catalytic reaction. This technique could not be used to study the Mn(II)-dependent activity of the adenylylated form of GS because of neglibible fluorescence changes accompanying the catalytic cycle. To circumvent this problem, GS was adenylylated in vitro with the fluorescent analog of ATP, 2-aza-1, ^{6}N-etheno AMP (aza-AMP) and by a spin labeled ATP derivative, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) AMP. With these derivatives, a detailed analysis of the reaction mechanism and topography around the adenylylation site could be made by fluorescence and epr techniques.

(i) Studies with Aza-GS. Ligand induced fluorescence changes associated with the binding of substrates to aza-GS were used to evaluate the thermodynamic and dynamic properties of the enzyme. From stopped-flow kinetic data, it was deduced that the biosynthetic reaction catalyzed by the Mn(II)-dependent aza-GS proceeds through a quaternary complex formed by the random binding of ATP, L-glu and NH_3, followed by sequential reactions involving at least four different fluorometrically detectable intermediates, I_1, I_2, I_3, and I_4. The rate determining step is the conversion of I_2 to I_3 which reacts with NH_3 to produce I_4. The data show that, in contrast to properties of the Mg(II) activated unadenylylated enzyme, there is no synergistic interaction of L-glu and ATP, and in the γ-glutamyl transfer reaction, L-glu antagonizes (rather than enhances) the binding of ADP and arsenate; however, ADP and arsenate enhance each others affinities for both enzyme forms. Tightening of the relaxed (metal-free) aza-GS by either Mn(II) or Mg(II) involves a very rapid step followed by a slow step ($t_{1/2} = 2$ minutes at 20°C).

(ii) Topography of the Regulatory Site. The environment of the adenylylation site of GS was monitored by measuring the ESR signal of the nitroxide radical on the Tempo-GS derivative. Distances between the nitroxide group at the adenylylation site and Mn(II) at the n_1 (structural binding site) and n_2 (the nucleotide binding site) were estimated to be 20 Å and 18 Å, respectively. In the presence of various substrates, the distance from the adenylyl group to the n_1 site is shortened, while that of the n_2 site is unchanged. By monitoring the ESR signal as a function of the Mn(II) concentration dissociation constants for the binding of Mn(II) to the n_1 and n_2 sites were determined to be 1.1 μM and 16.7 μM, respectively. These values agree well with the values of 1.8 μM and 18 μM determined by equilibrium dialysis. From a comparison of the ESR spectrum of free Tempo-ATP with that of Tempo-GS, it is deduced that the regulatory site of adenylylated GS is on the surface of the enzyme. This observation is in agreement with earlier conclusions based on NMR data (Proc. Natl. Acad. Sci. 75, 1255, 1978).

(iii) Studies with L-Methionine-S-Sulfoximine. by measuring fluorescence changes accompanying the binding of the R- and S-isomers of the transition state analog, L-methionine-S-sulfoximine, to the Mg(II)-unadenlylylated GS, it was found that the S-isomer exhibits an apparent negative cooperativity, whereas positive cooperativity was observed with the R-isomer. The cooperative effects are in accord with the existence of homologous subunit interactions in the fully unadenylylated enzyme. Moreover, the fact that the S- and R-isomers exhibit negative and positive cooperativity, respectively, suggests that the subunit interactions are sensitive to the absolute stereochemistry of the tetahedral sulfoximine moiety.

(b) Mechanistic Study of Alkaline Phosphatase: A Possible Physiological Role for Extreme Negative Cooperativity. The number of enzymes found to exhibit "half-of-the-sites" activity increases, but the physiological advantages of such phenomena remains unclear. In the case of alkaline phosphatase, it was proposed that the binding of the first substrate to the dimer greatly decreases the affinity of the other subunit for substrate, and that the binding energy is utilized to facilitate dephosphorylation of the phosphoenzyme intermediate. Although an attractive hypothesis, a detailed kinetic analysis of the reaction mechanism, has shown that the mechanism does not operate in the case of alkaline phosphatase. A plausible role for strong negative cooperativity in the regulation of alkaline phosphatase was disclosed by the discovery that Mg(II) can convert the low affinity substrate site to a high affinity site without affecting the affinity of the high affinity site. The net effect is to eliminate negative cooperativity in the response to increasing substrate concentrations. It follows that when substrate concentration is only high enough to saturate the high affinity sites, activity of the enzyme can be modulated over a wide range by changes in the concentration of Mg(II), and possibly other allosteric effectors. Thus, negative cooperativity, like positive cooperativity, provides a mechanism for the modulation of enzyme activity at fixed substrate levels by taking advantage of shifts from cooperative to hyperbolic substrate saturation isotherms in response to allosteric interactions.

(c) Activation of Cyclic Nucleotide Phosphodiesterase by Calmodulin. The binding of Ca(II) to calmodulin was determined by monitoring changes in the intrinsic fluorescence of calmodulin associated with Ca(II) binding, or by the use of dansylated troponin C to estimate the concentration of free Ca(II). The results show that calmodulin can bind up to four equivalents of Ca(II) by a mechanism that involves both positive and negative cooperativity. By means of a Job plot analysis and kinetic experiments, it was shown that calmodulin binds to two noninteracting sites on the dimeric cyclic nucleotide phosphodiesterase and that the activation constant of the diesterase for fully liganded calmodulin is about 1.0 nM. Based on the results of these studies and other considerations, it was proposed that activation of the phosphodiesterase involves a mechanism in which all forms of the Ca(II)-calmodulin, enzyme-calmodulin, and Ca(II)-calmodulin-enzyme complexes are possible intermediates. Although the parameters required to described the proposed mechanism are still under investigation, current results have revealed several important regulatory properties. They are: (i) The activated calmodulin-enzyme complex contains 4 equivalents of Ca(II); therefore, the activation of the phosphodiesterase becomes highly sensitive to the change in

Ca(II) concentration. (ii) The huge increase in affinity between calmodulin and enzyme derived from Ca(II) binding can be accounted for by a relatively small decrease in the dissociation constant for each of the 4 calcium binding steps. As a consequence, Ca(II) can be dissociated relatively rapidly from the calmodulin·Ca(II)·enzyme complex; hence the enzymic activity can be modulated rapidly in response to the changes in Ca(II) concentration.

c) Mechanism of Rabbit Skeletal Muscle Actomyosin ATPase. The Lynn-Taylor model for actomysin ATPase suggests that ATP hydrolysis requires the dissociation of ATP·actomysin complex to ATP·myosin and actin. However, results of studies using myosin subfragment-1 (S-1)(carried out in collaboration with E. Eisenberg) indicate that the acto-S-1 complex is not dissociated by ATP during the hydrolysis. Contrary to the Lynn-Taylor prediction, high actin concentration stimulates rather than inhibits ATPase.

B. Regulation of Enzyme Degradation

Balance between rates of synthesis and degradation governs the intracellular concentrations of enzymes. Since the relative concentration of different enzymes can change in response to variations in the nutritional state of the organism, highly specific regulatory mechanisms must control the differential synthesis and degradation of a given enzyme. Because protein turnover in bacteria shares at least two important features with turnover in mammalian cells, *i.e.*, selectivity and energy dependence, bacteria are convenient model systems in which to study this process.

(a) Glutamate Dehydrogenase (GDH) and Lysine-Sensitive Aspartokinase (AKIII). Of 18 enzymes examined in ammonia-starved *E. coli*, GDH and AKIII were most rapidly inactivated. That inactivation of these enzymes and general protein degradation share a common regulatory feature is indicated by the observations: (1) inactivation of both enzymes is prevented when protein degradation is blocked by either chloramphenicol, azide or PMSF; (2) inactivation does not occur during ammonia starvation in *relA* mutants in which protein turnover is blocked. Direct immunoprecipitation of GDH and AKIII from crude extracts using antibodies prepared against the purified enzymes demonstrated that degradation accompanies inactivation of both enzymes. Efforts to determine whether there is an inactivation step prior to degradation have yielded equivocal results. No inactivation of GDH could be detected in cell-free extracts, and efforts to demonstrate inactivation in cells permeabilized with toluene have yielded variable results. However, the discovery that incubation of purified GDH with NADPH leads to its rapid inactivation and renders it susceptible to proteolysis by various proteolytic enzymes, viz trypsin, chymotrypsin, subtilisin, thermolysis or pronase, suggests that this *in vitro* system may serve as a good model of how GDH degradation is regulated. The effect of NADPH is highly specific. Neither NADP, NAD, NADH, or other nucleotides destabilize GDH. Furthermore, the substrates α-ketoglutarate, glutamate, as well as several other anonic compounds can protect GDH from destabilization of NADPH.

The mechanism by which NADPH destabilizes GDH is still under investigation. It has been established that NADPH causes a perturbation of the ultraviolet spectrum of the enzyme and leads to exposure of one sulfhydryl group to rapid reaction with dithiobis-(nitrobenzoic) acid. Destabilization is not associated with a disappearance of NADPH, nor to a dissociation of aggregation of the enzyme.

(b) Degradation of Glutamine Synthetase. Under conditions of nitrogen starvation, the glutamine synthetase in catalase-deficient strains of Klebsiella aerogenes undergoes rapid inactivation and degradation. Results of studies with cell free extracts of this organism and partially purified enzyme preparations of E. coli suggest that the degradation of glutamine synthetase in these organisms involves two steps: First, the enzyme is inactivated by a catalase sensitive mixed function oxidase system; Second, the inactivated enzyme is proteolyzed. The inactivation reaction requires NADPH, and oxygen, and is stimulated by Fe(III). It is inhibited by chelating agents, Mn(II), catalase, and horseradish peroxidase.

A similar inactivation of homogeneous preparations of pure glutamine synthetase form E. coli is catalyzed by a highly purified cytochrome-P_{450} mixed function oxidase system from rabbit microsomes (kindly supplied by H. Gilboin and M. J. Coon), and also nonenzymically by model systems consisting of either ascorbate, O_2 and Fe(III), or of Fe(II), Fe(III) and O_2. After inactivation by either the microsomal-P_{450} system, or the ascorbate system, glutamine synthetase is readily degraded by a partially purified protease preparation from E. coli, whereas native (untreated) glutamine synthetase is not.

Preliminary experimental results indicate that the inactivation reaction may involve modification of a single tyrosine or a single histidine residue in each subunit of the enzyme.

The inactivation reaction may be the step at which glutamine synthetase degradation is regulated, since the inactivation of the unadenylylated (physiologically active) forms of glutamine synthetase is inhibited by the substrates glutamate and ATP, whereas these ligands stimulate the inactivation of the adenylylated (physiologically inactive) form of the enzyme. The inhibitory effects of catalase and Mn(II) on the inactivation reaction may also have a regulatory function.

C. Regulation of Enzyme Activity

(a) Glutamine Synthetase

(i) Studies with Permeabilized Cells. After freezing and thawing, exposure of E. coli cell suspensions to the nonionic detergent Lubrol WX leads to permeabilization of the cell membrane to small molecules, but not to proteins of greater than 6,000 molecular weight. Levels of the uridylyltransferase-uridylyl-removing enzyme (UT·UR) are about the same in cells obtained from nitrogen-starved and carbon-starved cultures. However, permeabilization of the nitrogen-starved cells leads to complete loss of UT·UR activity, whereas none of this activity is lost during permeabilization of carbon-starved cells. Permeabilization of either carbon- or nitrogen-starved cells leads to little or no loss of all other cascade enzymes, i.e., adenylyltransferase, P_{II} protein, and glutamine synthetase. It follows that permeabilized cells from nitrogen-starved cultures can be used to study metabolite regulation of the adenylylation-deadenylylation cycle alone, whereas permeabilized cells from carbon-starved cultures can be used to study the effects of metabolites on the complete bicyclic cascade system. Results to date show that allosteric effectors and substrates (i.e., glutamine, α-ketoglutarate, ATP, UTP, Pi, Mg(II)) which are known to affect the purified

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cascade enzymes in vitro have similar effects on the adenylylation and deadenylylation of endogenous glutamine synthetase in permeabilized cells. The steady-state level of glutamine synthetase adenylylation in the permeabilized cells varies in response to alterations of various metabolite concentrations of the suspending buffer, as would be expected from the in vitro experiments. It is concluded that important features of the cyclic cascade system which were disclosed by analysis of a theortical model and subsequently confirmed by in vitro experiments (see Curr. Topics Cell. Regul. 13, 1978) are operative also in the permeabilized cells.

(ii) Purification of the Uridylytransferase (UT) and Uridylyl-Removing (UR) Enzyme Activities. An E. coli strain (JA200/pLC 38-39) which carries the uridylyltransferase (UT) gene in a ColEl hybrid plasmid was shown previously to contain 25 times as much UT and UR enzymes as the wild type strain. Both activities were purified together throughout a 5,000-fold enrichment procedure that led ultimately to a homogeneous protein of 100,000 molecular weight. From its failure to dissociate under various denaturing conditions, it is concluded that the UR and UT activities are contained in a single polypeptide chain. This conclusion is in agreement with earlier genetic experiments showing that mutation leads to loss of both activities simultaneously, and that reversion leads to restoration of both activities. Now that the UT·UR enzyme has been obtained as a homogeneous protein, its interaction with the other cascade enzymes is open to investigation by both physical and enzymological approaches.

(iii) Multiple Molecular Forms of Glutamine Synthetase. From theoretical considerations, Raff and Blackwelder have shown that the GS in E. coli can exist in up to 384 different forms that differ from one another by the number (0-12) and distribution of adenylylated subunits. Although earlier studies showed the existence of multiple forms, efforts to separate them by electrofocusing, ion exchange chromatography, or electrophoresis failed. Now, by means of affinity chromatography on Affi-blue sepharose or on sepharose columns of immobilized anti-AMP antibodies, native enzyme preparations have been resolved into a series of more or less homogeneous fractions which contain different numbers of adenylylated subunits, ranging from 0 to 11 per enzyme molecule. In addition, multiple enzyme species containing the same number of adenylylated subunits, but which differ from one another in their affinities for ADP and/or sepharose-bound Cibacron dye, have also been obtained. These presumably represent isomeric forms that differ in the distribution of adenylylated and unadenylylated subunits within the dodecameric structure. The existence of isomeric forms is suggested also by results of immunotitrations showing that some species of adenylylated glutamine synthetase are precipitated by anti-AMP antibodies, whereas other species containing the same number of adenylylated subunits react with the antibodies to form soluble immune complexes.

(iv) Generation of Phosphotyrosyl Derivatives of Glutamine Synthetase. The recent discovery that proteins of mammalian cells contain phosphorylated tyrosine residues, and the demonstration that some viral infections are accompanied by the phosphorylation of protein tyrosyl groups, suggests that tyrosyl group phosphorylation may play an important role in cellular regulation. To aid in the development of reliable methods for the detection and quantitation of phosphotyrosyl residues in proteins, the adenylylated form of glutamine synthetase was treated with a nuclease that splits the adenylylated tyrosyl residues to yield free adenosine and the phosphotyrosyl protein derivatives.

(b) Regulation of Microtubule Assembly In vitro. Previous studies showed that in the presence of sodium fluoride, cAMP inhibits the assembly of microtubules in the supernatant fractions of crude pig brain homogenates. It is now found that adenosine, inosine, and guanosine, but not cytidine, stimulate the phosphorylation of proteins in the supernatant fraction. It is likely that in all cases inosine is responsible for the observed increase in protein phosphorylation, since (1) there is a lag in the time course of phosphorylation when adenosine is the activator, (2) adenosine is rapidly converted to inosine (and AMP) in these preparations, and (3) in the presence of EHNA, an inhibitor of adenosine deaminase, the effect of adenosine is abolished. Stability of the phosphoprotein bond is similar to that of acyl phosphates. Electrophoresis of the protein fractions in SDS polyacrylamide gel shows that inosine stimulates the phosphorylation of three proteins of approximately 63,000, 37,000, and 29,000 Daltons. The labeling patterns of these three proteins varies with the concentration of inosine used and the kind of divalent cation present. With Mg(II), the 29,000 Dalton protein is most heavily phosphorylated; with Ca(II), the 63,000 Dalton protein is preferentially phosphorylated, and in the presence of EDTA, only the 63,000 and 37,000 Dalton proteins are phosphorylated.

(c) Development of an Interconvertible Enzyme Cascade Model. A theoretical analysis of a monocyclic cascade model shows that the cyclic interconversion of an enzyme between covalently modified and unmodified forms has unusual regulatory capacities. In order to develop an in vitro system that can be used to test the validity of the theoretical predictions, a monocyclic system has been developed. The system consists of (1) the light chain from chicken gizzard myosin which serves as the interconvertible protein substrate; (2) a cyclic AMP-dependent protein kinase from bovine heart muscle; (3) a phosphoprotein phosphatase, also from bovine heart. All three components have been purified to near homogeniety. It has been established that the cyclic AMP-dependent protein kinase catalyzes phosphorylation of a single serine residue on the myosin light chain, and that this is probably the same serine that is phosphorylated by the cyclic AMP-independent kinase studied by other workers. The phosphoprotein phosphatase catalyzes the dephosphorylation of the phosphorylated light chain. Coupling of the kinase and phosphatase reactions should therefore provide an excellent model for studies on the regulatory characteristics of monocyclic cascades.

(d) Regulation of Rabbit Skeletal Muscle Actomyosin ATPase. Relaxation of skeletal muscle is thought to be mediated by the troponin-tropomyosin complex which binds to F-actin, and it has been suggested that in the presence of ATP and the absence of Ca(II), tropomyosin physically blocks the binding of the myosin cross-bridge to F-actin. Using a stopped-flow machine to monitor the binding of myosin subfragment-1 (S-1)·nucleotide complex to F-actin-troponin-tropomyosin complex (studies carried out in collaboration with E. Eisenberg), it was found that the troponin-tropomyosin complex exerts very little effect on the binding of S-1·ATP or S-1·ADP·Pi to F-actin. Thus, the troponin-tropomyosin complex does not inhibit actin-activated ATPase by preventing the binding of S-1·ATP or S-1-ADP to actin; instead it may inhibit the release of Pi from the acto-S-1·ADP·Pi complex.

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D. Branched Chain Amino Acid Metabolism

Earlier studies led to the discovery of a new pathway of leucine catabolism in bacteria. A key step in this pathway is the conversion of α-leucine to β-leucine which is catalyzed by leucine 2,3-aminomutase and is dependent upon the presence of adenosylcobalamin as a cofactor. The B_{12} coenzyme-dependent leucine 2,3-aminomutase is widely distributed in plant and animal tissues, and in microorganisms. Evidence has now been obtained suggesting that the pathway which serves a catabolic function in bacteria may have a biosynthetic role in animals. Cell-free extracts of rat liver catalyze the conversion of branched chain iso-fatty acids (isosterate, isomyristate, or isocaproate) to leucine. They also catalyze the conversion of valine, α-ketoisovalerate, or isobutyrate in the presence of acetate to leucine. These conversions are dependent upon added adenosylcobalamin and are inhibited by intrinsic factor as would be expected if the leucine mutase were involved. The branched-chain amino acid contents of sera from patients with several metabolic defects have been examined. Although the levels of branched-chain amino acids are elevated in patients with maple syrup urine disease, no β-leucine could be detected in the sera of these patients.

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Annual report of the
Section on Intermediary Metabolism and Bioenergetics
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

Continuing research activities of the investigators in the Section on Metabolism and Bioenergetics are primarily concerned with (1) the isolation and characterization of bacterial selenoenzymes and seleno-tRNAs, the mode of biosynthesis of these selenium-containing macromolecules and their biological functions; (2) characterization of 8-hydroxy-5-deazaflavin dependent enzymes of Methanococcus vannielii and the electron transfer roles of these enzymes in formate metabolism and methane biosynthesis and (3) phosphate esterification and electron transfer reactions catalyzed by enzymes from Clostridium sticklandii. Together these processes constitute important aspects of the overall problem of the mechanism of electron transport and energy conservation in the low potential range of biological redox systems.

A. Bacterial Seleno enzymes

(a) Nicotinic acid hydroxylase, an enzyme from Clostridium barkeri, catalyzes the first reaction in the fermentation pathway wherein nicotinic acid is ultimately converted to ammonia, carbon dioxide, acetate, and propionate (shown in earlier studies by E. R. Stadtman, I. Harary, L. Tsai, I. Pastan, J. Holcenberg). Later a rearrangement reaction step in the pathway was shown to be catalyzed by a new B_{12} coenzyme-dependent enzyme (Kung, Cederbaum, Tsai and T.C. Stadtman) and recently we have found that the first enzyme, the hydroxylase, is a selenoenzyme. Thus, this nicotinic acid fermenting anaerobic bacterium has proven to be a valuable source of enzymes that are specialized for the use of two nutritionally important trace elements - selenium and cobalt (in the form of Vit. B_{12}). In the conversion of nicotinic acid to 6-hydroxy nicotinic acid by nicotinic acid hydroxylase, NADP is the electron acceptor and is reduced to NADPH. An additional catalytic activity exhibited by the hydroxylase, in common with many other flavoproteins, is the dye-dependent oxidation of NADPH. From the relative effects of various inhibitors on the hydroxylase and oxidase activities of the enzyme it is deduced that the selenium moiety is located near or at the hydroxylation site and may not be required for the NADPH oxidase activity. The identity of the selenium moiety on the enzyme has not been determined but the ready release of ^{75}Se from the radioactive protein by reagents such as sodium dodecyl sulfate, thiols, urea, etc. indicate it is not a selenocysteine residue within a large polypeptide chain. Studies in progress are designed to isolate the ^{75}Se-labeled selenium compound after its release from the enzyme under controlled conditions and to determine its chemical properties.

(b) The most recently discovered selenoenzyme is thiolase which was isolated in the form of a ^{75}Se-labeled protein from Clostridium kluyveri cells that had been cultured in the presence of 1μM ^{75}Se-selenite and a large molar excess of sulfur. The total thiolase protein of C. kluyveri proved to be a mixture of two very similar 80,000 dalton enzyme forms; one of these, 5% or less of the total thiolase protein, contains selenium and the other (about 95%) is a selenium-independent thiolase. The selenoenzyme was separated from the non-selenium thiolase on the basis of its

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slightly greater affinity for DEAE-cellulose. Both thiolases then were isolated in essentially homogenous form by a variety of procedures including gel permeation chromatography, polyacrylamide gel electrophoresis and ammonium sulfate fractionation. Comigration of ^{75}Se and protein showing thiolase activity during electrophoresis in gels under non-denaturing conditions and comigration of ^{75}Se and protein subunits (40,000 daltons) in SDS-disc gel electrophoresis provided evidence of the presence of selenium covalently attached to the thiolase. The greater catalytic activity (five-fold or more) of the seleno-thiolase as compared to the non-seleno thiolase may be explained by the fact that a seleno-acyl ester intermediate on the seleno-enzyme would be much more reactive than a thiol-ester intermediate on the non-seleno enzyme form. Current studies have as their aim to identify the selenium moiety present in the seleno-thiolase and to establish the major biological role of this interesting enzyme. Seleno-thiolase, an enzyme which can catalyze in the biosynthetic direction the formation of a new carbon-carbon bond, provides the first example of a seleno-enzyme that is not a typical redox catalyst.

(c) Previously we have shown that selenium occurs as selenocysteine residues in polypeptide linkage in the selenium-dependent formate dehydrogenase of Methanococcus vannielii. Since it is likely that seleno-formate dehydrogenases from Escherichia coli also contain selenocysteine residues, we are now employing this organism as biological material to study the mechanism of biosynthesis of protein bound selenocysteine residues. If this amino acid is not introduced by way of a post translational modification of a preexisting amino acid residue (e.g. serine or cysteine) in the protein but instead is introduced while the polypeptide chain is synthesized (current claims of investigators working with glutathione peroxidase synthesis in rat liver) then we would expect to find as a minimum requirement a tRNA species that can be specifically charged with selenocysteine. The relatively greater stability of seleno-cysteine in crude enzyme preparations of E. coli as compared to those from Clostridium sticklandii, which we previously used for studies on selenoprotein biosynthesis, is one of the reasons for the present use of the E. coli system. Amino acyl tRNA synthetase preparations and amino acyl tRNAs have been prepared from E. coli cells and from C. sticklandii cells cultured under conditions where both organisms are actively synthesizing selenoproteins. The synthetase preparations from the two different organisms exhibit similar activities when tested with their homologous or heterologous bulk tRNAs as acceptors for mixtures of amino acids. Hence, tRNA populations from both organisms can be examined with the E. coli synthetase preparation for the possible presence of a specific selenocysteine accepting species.

(d) Continued studies on Glycine reductase selenoprotein A (M_r12,000) have dealt mainly with the production of ^{75}Se-labeled protein for selenopeptide analysis and for use in in vitro ^{32}P-labeling experiments. If a phosphate ester of the selenol group or one of the thiol groups of seleno-protein A is the phosphate ester intermediate generated concomitant with glycine reduction to acetate by glycine reductase then it should be possible to find conditions where the ^{32}P-labeled seleno-protein is detectable. To date trace amounts of low molecular weight ^{32}P-proteins(s) have been detected electrophoretically when the glycine reductase reaction

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is catalyzed by crude enzyme preparations in the presence of labeled phosphate. The experiments are preliminary to those in which a wide variety of conditions will be explored and finally substrate amounts of enzymes will be employed.

Studies on the biosynthesis of selenoprotein A show that once the biologically active ^{75}Se-protein is formed, it is stable and remains in the cells even during prolonged incubation in the presence of antibiotics such as chloramphenicol, rifampicin, etc. that prevent cell multiplication. No stable precursor of the protein has been detected in selenium-deficient cells.

B. Bacterial Seleno-tRNAs

Amino acid transfer nucleic acids (tRNAs) modified in the polynucleotide portion of the molecules with selenium were first detected in *C. sticklandii*. Seleno-tRNAs also occur in other anaerobic microorganisms but *C. sticklandii* is the richest source found so far. One strictly aerobic organism, a Pseudomonas, grown in the presence of ^{75}Se-selenite formed several ^{75}Se-labeled tRNA species and one of these copurified with leucine accepting activity.

^{75}Se-labeled tRNAs from *C. sticklandii* were converted to populations of ^{75}Se-nucleotides by mild alkali digestion and the ^{75}Se-labeled products were separated chromatographically and analyzed electrophoretically and by thin layer chromatography. Satisfactory recovery of ^{75}Se-labeled nucleotides indicates that the procedures developed may be adequate for final isolation and identification of the selenobase(s) present.

C. Proline reductase

Oxidation of certain amino acid substrates in *C. sticklandii* is coupled via pyridine nucleotides and electron carrier proteins to the reduction of glycine and proline which serve as important terminal electron acceptors for the organism. A 250,000 dalton iron protein that is a component of the electron transport chain linking NADH and proline reductase was isolated in homogeneous form and shown to be glycoprotein. The glycosyl portion is made up of three different sugars, one of which seems to be xylose. No amino sugars were detected. In view of the fact that proline reductase is a membrane protein interaction with the iron protein, its immediate electron donor, presumably is facilitated by the presence of glycosyl groups on the latter. The amino acid composition of the iron protein is typical of many proteins. There is one gram atom of iron per mole of protein (250,000 M_r).

D. 5-deazaflavin-dependent reactions

A novel cofactor, 8-hydroxy-5-deazaflavin, that is abundant in methane-producing bacteria serves as electron carrier in coupled oxidation reactions wherein formate (via formate dehydrogenase) or molecular hydrogen (via hydrogenase) is the electron donor substrate.

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A dehydrogenase that specifically utilizes the deazaflavin as cofactor then reacts with a pyridine nucleotide ($NADP^+$) and generates the reduced pyridine nucleotide. The mechanism of the 5-deazaflavin-dependent enzyme mediated reaction involves the direct transfer of hydrogen between the 5-deazaflavin and $NADP^+$. The stereochemistry of this direct H-transfer process, studied in both the forward and reverse reactions with ^3H-specifically labeled 5-deazaflavin$\cdot H_2$ or NADPH, showed it to be S-specific with respect to $NADP^+$. In careful pH studies the nature of the reactive deazaflavin species has been determined in both the forward and reverse reactions.

Homogeneous preparations of the 5-deazaflavin dependent $NADP^+$ reductase from Methanococcus vannielii contain 2 gram atoms of zinc per mole. Half of the zinc can be readily removed with concomitant loss of 50% of enzymic activity. Although naturally occurring flavins such as FMN and FAD do not serve as cofactors for the enzyme a few of the synthetic deazaflavin analogs of the natural 5-deazaflavin cofactor that have been prepared exhibit limited cofactor activity. The binding properties of these or other analogs will be tested in order to design a derivative suitable for affinity chromatography. Using an immobilized 5-deazaflavin chromatographic reagent it will then be possible to examine crude extracts of methane-producing bacteria for other enzymes that employ the deazaflavin as cofactor. Enzymes involved in the overall pathway of CO_2 reduction to CH_4, most of which are still unknown, could be among the logical candidates to also employ the abundant 5-deazaflavin as cofactor.

Annual Report of the
Section on Protein Chemistry
Laboratory of Biochemistry
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

Research in the Section on Protein Chemistry consists of studies on the physical and chemical properties of macromolecules of biological interest and on the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation.

Glutamine synthetase, a strictly regulated enzyme in Escherichia coli, is a dodecamer with twelve catalytic sites. Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from E. coli have been studied by microcalorimetry, equilibrium dialysis, pH, ultracentrifugal, spectral, and kinetic techniques. Calorimetrically measured heats provide information on the separateness of ligand binding sites and on proton uptake or release in binding reactions. Thermal saturation curves for the binding of low affinity ligands to the enzyme give thermodynamic binding parameters. A reversible thermal transition (observed by UV difference spectral measurements) is being used to study Me^{2+} and ligand stabilization of the glutamine synthetase structure. The thermal transition occurs at high temperature over a narrow temperature range and appears to involve the exposure of one catalytically essential tryptophanyl residue per subunit with parallel changes in catalytic activity; subunit dissociation does not occur during the annealing process. Spectrophotometric and sedimentation velocity measurements show that unadenylylated and adenylylated glutamine synthetase from E. coli undergo different conformational changes on binding L-methionine-SR-sulfoximine, a proposed transition state analog. The binding of this analog produces both local and gross conformational differences between unadenylylated and adenylylated enzymes. Calorimetric and UV spectrophotometric studies are being conducted with the resolved S and R diastereoisomers of L-methionine-SR-sulfoximine in order to characterize interactions at the subunit catalytic site with substrates and subunit interactions within the dodecamer. UV spectral changes produced by the S- and R-isomers of this analog are essentially identical, indicating that both isomers produce a similar local perturbation of aromatic residues on binding. However, the enzyme has an approximately 10-fold greater affinity for the S- than for the R-isomer. Subunit interactions appear to be prevalent when ~ 50% of the subunit catalytic sites are filled with the S isomer. Calorimetric measurements show that the heat for binding the S-isomer to the Mn-enzyme is about twice as negative as that for binding the R-isomer; proton effects on binding these diastereoisomers also are quite different. Preliminary results with a 1:1 mixture of the S- and R-isomers suggest that some of the heat and proton uptake produced by binding the S-isomer to the Mn-enzyme can be attributed to subunit interactions. In related studies, L-glutamate and L-glutamine have been found to produce different UV spectral perturbations on binding to unadenylylated and adenylylated Mn enzymes.

A novel reaction has been discovered to be catalyzed by unadenylylated, manganese glutamine synthetase: an AMP-dependent (reversible) synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine. The synthesis of pyrophosphate is coupled to the hydrolysis of the amide bond of L-glutamine. In this reaction, Mg^{2+} will not substitute for Mn^{2+} and the fully adenylylated Mn-enzyme is inactive. Thus, under assay conditions, the conformations of the Mn^{2+}- and Mg^{2+}-unadenylylated enzymes and of the Mn^{2+}-unadenylylated and Mn^{2+}-adenylylated enzymes must differ. Pyrophosphate synthesis relates to the mechanism of reactions catalyzed by glutamine synthetases from various sources, and shows that the nucleotide site must be filled for synthesis of the β,γ phosphate bond, which results in PP_i or ATP synthesis if AMP or ADP, respectively, is the supporting nucleotide. The AMP-supported synthesis of PP_i and L-glutamate clarifies the role of AMP in related reactions catalyzed by the unadenylylated, manganese enzyme; namely, AMP-supported arsenolysis of L-glutamine and AMP-supported γ-glutamyl transfer. The catalytic site of glutamine synthetase has a rather broad specificity for the nucleotide base, since GMP and IMP also support the last two reactions.

In continuing studies of metal ion interactions with glutamine synthetase, we have explored the use of xylenol orange (a Mn^{2+} binding dye), pyridine-2,6-dicarboxylic acid (dipicolinic acid), and pH indicator dyes. The results demonstrate the general usefulness of these reagents in studies of protein-Mn^{2+} interactions. Our studies show that Mn^{2+} binding to n_1 structural sites of glutamine synthetase promotes a transition from a low- to a high-affinity conformation. In this process, there is a pK_a perturbation (synchronous with the protein conformational change) of an ionizable amino acid residue that is not involved in binding Mn^{2+}. Mn^{2+} dissociation from the enzyme is first order with a half-time of 0.22 second at 15°C, as measured in a stopped-flow apparatus with xylenol orange. Experiments with dipicolinate (in the absence and presence of bromothymol blue) verifed the off-rate of Mn^{2+} from the enzyme and showed that there is a fast proton uptake coincident with Mn^{2+} dissociation from the protein. Thus, one proton equivalent appears to bind at the rate of Mn^{2+} dissociation (presumably to a group within the Mn^{2+} binding cluster) and another proton equivalent per subunit binds after Mn^{2+} release as the protein undergoes the conformational transition.

The studies on aspartate transcarbamoylase (ATCase) from E. coli (in collaboration with H.K. Schachman at the University of California, Berkeley, California) are completed and give an estimate of the enthalpy change for the substrate-promoted conformational transition ($\Delta H_{T \rightarrow R}$) of ATCase. We used two calorimetric approaches and a high-affinity bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA) for binding to ATCase to promote the conformational transition of the enzyme; (1) Δ (ΔH) measurements of PALA binding to ATCase at different stages of the conformational transition (monitored by the difference sedimentation technique) in $K\text{-}PO_4$ and $K\text{-}PO_4$/TES buffers at pH 7.0 and 30°C allow calculation of the heat of the conformational transition from an initial T state to a final R state ($\Delta H_{T \rightarrow R}$), the intrinsic heat of binding PALA (ΔH_{PALA}), and a measure of proton uptake or release associated with the conformational change and the binding of PALA. (2) The heats of assembly of catalytic trimers (C) and regulatory dimers (R) into ATCase (C_2R_3) in the absence and presence of PALA, the heat of binding PALA to the isolated catalytic subunit (C) of ATCase, and the value of ΔH_{PALA} from (1) give another estimate of $\Delta H_{T \rightarrow R}$. The two approaches in (1) and (2) give: $\Delta H = -6 \pm 3$ and -14 ± 9 kcal/mol of ATCase, respectively, at pH 7.0 and 30°C; a proton uptake of 2 to

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to 6 equivalents of H^+ per mol of ATCase accompanies the protein conformational change. With $\Delta G'_{T \to R}$ = + 3.3 kcal/mol of ATCase (Howlett, G.J., Blackburn, M.N., Compton, J.G., and Schachman, H.K., Biochemistry 16, 5091-5099, 1977) and the best estimate of $\Delta H_{T \to R}$, $\Delta S_{T \to R}$ = -31 cal (deg·mol of ATCase)$^{-1}$ and $-T\Delta S$ = + 9 kcal/mol. Thus, the T→R transition of ATCase is entropically controlled at 30°C. Sedimentation analyses of assembly reactions were consistent with the binding of PALA to C subunits causing a weakening of regulatory and catalytic interchain contacts. From the heats of subunit assembly in the presence and absence of PALA (-54 and -76 kcal/mol of enzyme, respectively), the PALA-linked subunit interaction enthalpy change is + 22 ± 8 kcal/mol of ATCase, indicating that the corresponding entropy change is positive ($\leq$ + 80 cal/deg· mol).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00201-09 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism of the Branched-Chain Amino Acids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. M. Poston	Research Chemist	LB NHLBI
Others:	None		

Cooperating Units

Dr. Ingrid Lombeck, University of Duesseldorf, Duesseldorf, West Germany
Dr. Isamu Matusmoto, Kurume University, Kurume, Japan
Dr. Geraldine Schechter, Veterans Administration Hospital, Washington, D.C.
Dr. Rose Sheinin, University of Toronto, Toronto, Ontario, Canada
Dr. Göran Steen, University of Göteborg, Göteborg, Sweden
Dr. H. Boehles, University Children's Hospital, Erlangen, West Germany

~~COOPERATING UNITS (if any)~~
Dr. Richard Hillman, Washington University, St. Louis, Missouri

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A study of the metabolism of the branched-chain amino acids has revealed a pathway of metabolism of leucine that is catabolic in bacteria and appears to be synthetic in humans. The pathway depends upon the activity of the enzyme, leucine 2,3-aminomutase, which requires adenosylcobalamin as a cofactor. Leucine may be synthesized from iso-fatty acids or from the catabolites of valine. The B_{12}-dependent enzyme, leucine 2,3-aminomutase, is stimulated by FAD, coenzyme A, NAD, and pyridoxal phosphate. The relationship between enzyme activity and various disease states such as pernicious anemia and maple sugar urine disease will be examined.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The catabolism of the branched-chain amino acids, leucine isoleucine, and valine remains incompletely understood. Although the study of certain inborn errors of metabolism, *e.g.*, maple syrup urine disease and isovaleric acidemia, has given some insight into the metabolism of these amino acids, additional information has been derived from studies of bacterial fermentation. The objectives of this project are to establish the fermentation pathways of leucine and the other branched-chain amino acids, to examine the enzymes responsible for the various metabolic steps in these fermentations, to explore the distribution of these pathways in other species, and to examine the implications of these pathways in human metabolism.

Major Findings

As reported previously, when cells or extracts of several clostridia, especially *Clostridium sporogenes* and *Clostridium lentoputrescens*, are incubated with L-leucine, several metabolic products are formed that are consistent with the metabolic pathway reported in mammals. However, the production of isobutyrate could not be explained by previously postulated pathways. Evidence was accumulated to support the pathway:

α-leucine → β-leucine → β-ketoisocaproate → acetate + isobutyrate

The first step is catalyzed by the enzyme, leucine 2,3-aminomutase, which requires coenzyme B_{12} [adenosylcob(III)alamin]. This enzyme has been partially purified from a variety of sources: clostridia, spinach, potatoes, and sheep liver. It has been measured in human liver, hair roots, and leukocytes.

Certain temperature sensitive mutant cell lines of mouse L cells have been proposed as animal models for megaloblastic anemia. In cooperation with Dr. Rose Sheinin of the University of Toronto, this proposed model was examined for leucine 2,3-aminomutase and for its response to adenosylcobalamin. The studies begun last year and completed since the last report clearly show that there was no correspondence between the level of activity of the enzyme and the addition of adenosylcobalamin; nor was there any correspondence between the level of enzymic activity and the morphological traits on which the model was proposed. While it is unfortunate that a good model has not been found, it is valuable to know that, however accurate the mouse L cell model may be with regard to DNA synthesis and cellular morphology, *ts* A1S9 and *ts* C1 cell lines are essentially normal with regard to their B_{12} metabolism. The DNA and morphology characteristics must arise from a B_{12}-independent lesion.

Studies initiated last year as a result of the finding that patients with untreated pernicious anemia have elevated serum β-leucine levels have been continued. Since such patients are deficient in B_{12}, the enzyme leucine 2,3-aminomutase is probably inactive. In such circumstances, leucine is probably not the source of the β-leucine and another source must be established. At least two possibilities exist. The branched-chain carbon skeleton can be provided by beta oxidation of fatty acids of the iso-series; the β-ketoisocaproate produced can be transaminated to yield β-leucine. The other possibility is that valine can be catabolized to isobutyrate or isobutyryl-coenzyme A which can condense with acetate to yield β-ketoisocaproate.

It has been shown that when cell-free extracts of rat liver are incubated with the iso-fatty acids, isostearate, isomyristate, or isocaproate, there is a substrate-dependent production of leucine. This production is stimulated by the addition of adenosylcobalamin and inhibited by intrinsic factor -- conditions expected if the leucine 2,3-aminomutase functions in this production. Moreover, incubation of the extracts with valine or with the metabolites of valine, α-ketoisovalerate or isobutyrate, in the presence of acetate also leads to the substrate-dependent production of leucine. This production, too, is stimulated by adenosylcobalamin and inhibited by intrinsic factor. These findings are consistent with the idea that the pathway through β-leucine may function in a synthetic direction. Similar results were obtained when the incubations were conducted with extracts of human leukocytes or of human hair roots (bulb and sheath). Not only was there a substrate-dependent production of leucine by human leukocytes, but when the extracts were incubated with radiolabeled isobutyrate, it was possible to isolate radiolabeled leucine as a product of the incubation. This demonstrates that the carbon chain, itself, is directly synthesized from these fatty acid precursors.

Several sera from patients with metabolic defects have been examined during the year with regard to their branched-chain amino acid content. Most notably it has been found that even though the levels of leucine, isoleucine, and valine are greatly elevated in cases of maple syrup urine disease, there is no detectable β-leucine in the serum of these patients. And this is so even though the B_{12} content of the serum of the patients is normal. This suggests that a lesion may exist in leucine 2,3-aminomutase in maple syrup urine disease. It has not yet been possible to obtain either leukocytes or tissue samples to assay the enzyme directly.

Initial attempts at establishing the intracellular locus of leucine 2,3-aminomutase suggest that the enzyme is in the cytosol, principally, of rat liver. This contrasts with the mitochondrial locus of the branched-chain amino acid dehydrogenases, most of which is extrahepatic.

As mentioned, leucine 2,3-aminomutase has been measured in hair roots. This is a relatively easily obtained tissue and should lend itself to sampling of large populations for enzyme activity. The activity seems to survive quite well in roots that have been taped to a card and allowed to remain at room temperature for several days. This suggests that samples of hair roots can be sent by mail with little effect on the outcome of the assay.

Proposed Course of Action

The leucine mutase will be purified and characterized. To this end, the conditions which yield maximum activity in cell cultures will be established, and sources which may yield stable enzyme will be examined. The nature of the B_{12} involvement will be established and the nature of the other cofactor involvement will be examined. The distribution of the pathway in normal and diseased mammalian and human tissue will be studied as will its relation to plant development. The relation of the pathway to the metabolism of other amino acids and to lipids will be examined. The other enzymes in the pathway will be examined and their cofactor requirements established.

Relevance to Biomedical Research

This study impinges on at least three areas of medical concern: (1) the mode of action of vitamin B_{12} in its metabolic roles; (2) the means by which organisms catabolize food materials; and (3) the probable nutritional value of plant material with regard to vitamin B_{12}. The second area is directly concerned with several inborn errors of metabolism that have been shown to be devastating to the well being of humans, especially in the instances of maple syrup urine disease, isovalericacidemia, and disorders of the catabolism of short-chain acids. The mode of action of B_{12} is imperfectly understood, but its importance in hematapoiesis and in the maintenance of proper neurological function is exemplified in the disease of its metabolic deficiency, pernicious anemia.

Publications

Poston, J. Michael, and Hemmings. Brian A.: Cobalamins and cobalamin-dependent enzymes in Candida utilis. J. Bacteriol. 140: 1013-1016, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00202-09 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Kinetics, Regulation and Mechanism of Biochemical Reactions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. B. Chock	Research Chemist	LB NHLBI
	Vincent Chau	Staff Fellow	LB NHLBI
Others:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	Sue Goo Rhee	Research Chemist	LB NHLBI
	Charles Y. Huang	Research Chemist	LB NHLBI
	J. H. Wang	Expert Consultant	LB NHLBI

COOPERATING UNITS (if any)
Emily Noiman, Johns Hopkins University, Baltimore, Maryland
Gregory Ubom, The Catholic University of America, Washington, D. C.
Evan Eisenberg, Laboratory of Chemistry, NHLBI, NIH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.5	3.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) Study of E. coli alkaline phosphatase reveals a possible regulatory role for negative cooperativity. It provides a means for a rapid surge or decrease in enzymic activity through changes in the concentration of an allosteric effector such as Mg(II) which can practically desensitize the negative cooperativity. (2) Mechanistic study of 2-aza-ε-adenylylated glutamine synthetase (GS) reveals the catalytic cycle for the adenylylated GS. Topographical information on the adenylylation and catalytic sites and substrate induced conformational changes of the adenylylated enzyme were obtained using a spin-labeled Tempo-adenylylated GS. Results from fluorescence and polarization studies on the binding of L-methionine-S-sulfoximine and its R-isomer to the Mg(II) activated unadenylylated GS confirm the homologous subunit interaction in GS. (3) Mechanistic study on the activation of cyclic nucleotide phosphodiesterase by calmodulin may reveal a general mechanism for calmodulin activation of enzymic activity. (4) Theoretical analysis of cyclic cascade model and the study of adenylylation-deadenylylation of the GS system and phosphorylation-dephosphorylation of protein are being continued. The mechanism and regulation of actomycin ATPase were investigated.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: (1) Utilizing the physical-chemical approach to study the kinetics, mechanism, and regulation of biochemical activity. In particular, the biochemical action between the substrates, metabolites, and enzymatic activity of glutamine synthetase from Escherichia coli will be elucidated. In addition, fast kinetic techniques which are useful in these studies will be improved. (2) Theoretical analysis of the cyclic cascade system with respect to its properties and function in the metabolic regulation of key enzymes will be continued. (3) Isolation of the regulatory proteins to allow detailed mechanistic studies, and experimentally verify the validity of the cyclic cascade model described in (2). (4) To study the regulatory mechanism of calmodulin activated cyclic nucleotide phosphodiesterase.

Major Findings

I. Mechanistic Study of Alkaline Phosphatase: A Possible Physiological Role for the Extreme Negative Cooperativity. With the increasing number of enzymes reported to exhibit strong negative cooperativity, it is inevitable for one to question the physiological role or roles of this extreme form of negative cooperativity. Nearly ten years ago, based on the study of E. coli alkaline phosphatase, Lazdunski and his coworkers conferred a possible role for the idle subunit of this strong negative cooperative enzyme (Eur. J. Biochem. 20:124, 1971). E. coli alkaline phosphatase is a dimeric enzyme consisting of two identical subunits. At alkaline pH, both phosphate binding and catalysis have been shown to proceed via a strong negative cooperativity mechanism. The flip-flop model proposed by Lazdunski suggests that the binding of the first substrate to the dimer greatly decreases the affinity of the other subunit for substrate. The formation of the phosphorylated enzyme intermediate then leads to regain the substrate binding capacity of the second subunit. The binding energy is utilized to facilitate the dephosphorylation reaction. In this mechanism, the direct phosphate hydrolysis step is assumed to be negligible, compared with the substrate-facilitated pathway. With the flip-flop model, the catalysis would yield a normal Michaelis-Menten kinetic response, because the second power terms in the initial rate equation for the flip-flop model can be canceled to give a Michaelis-Menten type of kinetic expression. In order to differentiate the flip-flop model from a simple mechanism, which does not involve the substrate-facilitated product release step, an alternative substrate method was used, because in the presence of an alternative substrate, the initial rate equation derived for the flip-flop model contains noncancelable second power terms; whereas with the simple model, the alternative substrate simply behaves like a competitive inhibitor. Therefore, in the presence of an alternative substrate, one would expect a nonhyperbolic kinetic pattern for the flip-flop model and a hyperbolic kinetic pattern for the simple model. Experiments carried out with CMP as alternative substrate and 6-bromo-2-hydroxy-3-naphthoyl-O-anisidine phosphate as substrate showed that the results are contradicted with the flip-flop model, but is consistent with the simple model. To avoid the possibility that the expected nonlinear plot expected for the flip-flop model may not be readily detectable within the experimental conditions, we made use of the constant-ratio alternative substrate approach. Under these conditions, one would expect linear double reciprocal plots for both the flip-flop model and the simple model; but when the alternative substrate to substrate ratio is varied, one expects to obtain a family of intercepting lines for the flip-flop model, whereas the simple model will yield a family of parallel lines. The data obtained for this type of experiment yielded only parallel lines. Thus, the results are in accord with the simple model. Furthermore, since the unassisted phosphate release step was ignored in the flip-flop model, the substrate facilitated phosphate release step has to be at least ten times faster than the unassisted step. However, the value of k_{cat} determined from steady-state kinetics is in agreement with the off-rate of inorganic phosphate measured in the absence of substrate using the ^{31}P NMR method (Biochemistry 15:1547, 1976). If k_{cat} and the "off-rate" of phosphate are of the same magnitude, then there is no reason for the existence of the substrate-facilitated pathway, unless binding of substrate will facilitate the conversion of the covalently bound phoshate to the noncovalently bound enzyme-phosphate complex. To explain this possibility, the kinetics of the dephosphorylation was measured using a 3-syringe stopped-flow machine. The results show that the dephosphorylation step is about ten times faster than k_{cat}, and its rate is unaffected by the presence of substrate or inorganic phosphate.

Therefore, flip-flop is not operative in alkaline phosphatase. To search for another possible role for the strong negative cooperativity, we investigated the possibility of desensitization of this strong negative cooperativity by an allosteric effector. In this aspect, we found that Mg(II) can shift the high K_m site to a low K_m site. The value of this high K_m is shifted from 260 μM to 5 μM in the presence of Mg(II). However, Mg(II) exerts essentially no effect on the low K_m site. In other words, Mg(II) can practically desensitize the strong negative cooperativity of alkaline phosphastase. Based on the observation with Mg(II), we propose that strong negative cooperativity, at least in the alkaline phosphatase case, can provide a regulatory role. It provides a means for a rapid surge or decrease in enzymic activity through changes in the concentration of an allosteric effector such as Mg(II). In contrast, positive cooperativity provides a relatively large change in enzymic activity when the substrate concentration is varied within a relatively small range.

II. Fluorescence Binding Study of L-Methionine-S,R-Sulfoximine: Additional Evidence Supporting Subunit Interaction in Unadenylylated Glutamine Synthetase (also see Annual Report of S.G. Rhee). We have previously demonstrated that the rate of irreversible inactivation of the unadenylylated glutamine synthetase by the L-methionine-S-sulfoximine slows down progressively from the expected first order rate. This observation indicates that an inactivated subunit in the dodecameric enzyme retards the reactivity of its neighboring subunits toward methionine sulfoximine and ATP. In this study, we demonstrated that upon addition of the highly resolved L-methionine-S-sulfoximine or its R-isomer to the Mg(II) activated unadenylylated glutamine synthetase in the absence of nucleotide, the protein tryptophan fluorescence was enhanced significantly. Both the S-isomer and R-isomer induced a fluorescence increase to about 65% of the initial protein fluorescence intensity. This is significantly different from 5% decrease in protein fluorescence observed due to the binding of either L-glutamate or L-glutamine to the

Mg(II) activated unadenylylated enzyme. These results indicate that the L-methionine sulfoximine diastereomers stabilize a distinctively different conformational state of the Mg(II) activated enzyme than that associated with the binding of L-glutamate or L-glutamine. The difference is further confirmed by the fact that in the presence of AMPPNP, both S- and R-isomers cause a 15% increase in protein fluorescence, whereas no fluorescence change was observed when glutamate was added under similar conditions. Binding of the S-isomer also causes a significant change in fluorescence polarization as monitored with the tryptophan signal of the protein. The polarization decreased from 0.54 to 0.47 when the enzyme was 90% saturated with the S-isomer. With the addition of ATP (1.0 mM) to this mixture, the enzyme was completely inactivated due to the formation of tightly bound ADP and methionine sulfoximine phosphate, and the polarization was further decreased to 0.44. A similar decrease in fluorescence polarization was also observed when the enzyme was saturated with ATP and glutamate.

The fluorescence changes induced by the S- and R-isomer were used to monitor the binding of these ligands. The results show that the binding isotherm for the S-isomer exhibits an apparent negative cooperativity with a Hill's number equal to 0.65 and $[S]_{0.5}$ = 8.7 mM. With the R-isomer as ligand, an apparent positive cooperativity was observed with $n_H = 1.3$ and $[S]_{0.5}$ = 7.1 mM. In the presence of AMPPNP, the value of $[S]_{0.5}$ for the S-isomer is reduced to 0.4 mM; whereas the apparent negative cooperativity remains unchanged with $n_H = 0.7$. Both the positive and negative cooperativities observed are in accord with the existence of homologous subunit interaction in the fully unadenylylated enzyme. Since the negative and positive cooperativities were detected for S- and R-isomers, respectively, it suggests that the subunit interaction is sensitive to the absolute stereochemistry of the tetrahedral sulfoximine moiety.

III. Mechanistic Studies of 2-Aza-ε-Adenylylated Glutamine Synthetase (also see Annual Report of S.G. Rhee). The catalytic cycle for the biosynthesis of glutamine catalyzed by the unadenylylated glutamine synthetase from *E. coli* has been elucidated utilizing the intrinsic tryptophan fluorescence of the protein (Proc. Natl. Acad. Sci. 73:476, 1976). However, due to the lack of detectable signal changes accompanying the catalytic process, the detailed mechanistic action of the adenylylated enzyme has yet to be investigated. In this study, the enzyme was adenylylated with a fluorescence derivative of ATP, 2-aza-ε-ATP. With this ATP analog, one can adenylylate the enzyme up to 10 2-aza-ε-AMP per dodecamer. The 2-aza-ε-adenylylated enzyme exhibits similar catalytic properties to those of the naturally adenylylated enzyme (see Table I).

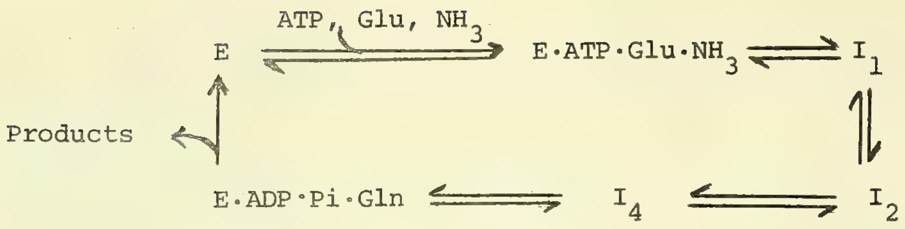
The fluorescence spectrum of 2-aza-ε-adenylylated enzyme exhibits two excitation maxima at 300 nm and 368 nm, and the emission maximum is at 470 nm. The emission quantum yield is dependent on the excitation maximum used and is sensitive to the conformational changes induced by the binding of divalent metal ion, and substrate, and by the formation of reaction intermediates. These changes in fluorescence intensity were utilized to monitor the tightening reaction of relaxed aza-adenylylated-GS, the binding of substrates to the Mn(II) activated aza-enzyme for both the transferase reaction (ADP, Gln,

Table I. Comparison of the Catalytic Properties of the 2-Aza-ε-Adenylylated and Naturally Adenylylated Enzymes

		naturally adenylylated GS	2-aza-ε-adenylylated GS
Mn(II) supported activity		+	+
Mg(II) supported activity		-	-
pH for maximum activity		6.7	6.3
K_m for:	ATP	33 μM	50 μM
	Glu	5 mM	6 mM
	NH_4^+	0.1 mM	0.13 mM
Relative V_{max}		1.0	1.3

arsenate), and the biosynthetic reaction (ATP, Glu). Binding of NH_2OH and NH_3 do not induce any significant change in fluorescence intensity, except when the enzyme is saturated with ADP, Asi and Gln, or ATP and Glu, respectively. The results obtained at pH 7.2 indicate that ATP, Glu, ADP, Gln, and arsenate can bind to the enzyme with a random order mechanism. The binding constants indicate that arsenate antagonizes the binding of Gln, while it exhibits a strong synergistic effect with ADP binding, and ADP exerts no effect on Gln binding. In the biosynthetic system, no synergistic effect was observed between ATP and Glu. This is different from the strong synergistic effect between ATP and Glu observed for the Mg(II) activated unadenylylated enzyme. It is noteworthy that addition of ATP and Glu to the catalytically inactive Mg(II) bound aza-enzyme only causes a small change in fluorescence intensity, and no fluorescence change was detected due to the binding of NH_3 to the Mg(II)·aza-GS·ATP·Glu complex. The rate of the tightening reaction induced by the binding of either Mg(II) or Mn(II) to the divalent-metal-free aza-GS was monitored by the fluorescence change (excited at 368 nm). The results show that the same rate was obtained with either Mg(II) or Mn(II), and the reaction proceeds with a very rapid phase followed by a slow step which has a $t_{1/2}$ of 135 seconds at 20°C in a pH 7.2 buffer containing 50 mM Hepes-KOH and 0.1 M KCl.

A rapid kinetic technique was used to elucidate the catalytic cycle for the biosynthesis of glutamine catalyzed by the Mn(II) activated 2-aza-ε-adenylylated enzyme. All the experiments were carried out at 10°C in a pH 7.2 buffer solution containing 50 mM Hepes-KOH, 0.1 M KCl, and 1 mM $MnCl_2$. The reactions were monitored with a stopped-flow fluorometer for the change of fluorescence intensity at 470 nm setting the excitation wavelength at either 300 nm or 368 nm. The rate was measured for the reaction of various combinations of enzyme-substrate complex with various substrates. The data suggest that the catalytic cycle can be described by the following scheme.



where E represents the Mn(II) activated aza-adenylylated GS and I_n is fluorescent distinct reaction intermediates. The rate limiting step in this cycle is the formation of an activated E'·ATP·Glu complex (I_3). The rate constant for this step is ~ 0.43 sec^{-1} which is in reasonable agreement with the k_{cat} of 0.5 sec^{-1} obtained from the steady-state assay.

IV. Study of 2,2,6,6,-Tetramethylpiperdine-1-oxyl (Tempo) Adenylylated Glutamine Synthetase by the ESR Method (also see Annual Report of S.G. Rhee). Spin-labeled ATP was used to adenylylate the E. coli glutamine synthetase. The adenylylation reaction was carried out until 7 Tempo-AMP was attached to each dodecameric enzyme. The Tempo-AMP adenylylated enzyme exhibits similar catalytic properties, pH profile, and inhibitor susceptibility to those of the naturally adenylylated enzyme. With this modified enzyme, it is possible to monitor the environment of the adenylylation site of glutamine synthetase by measuring the ESR signal of the nitroxide radical on the Tempo-AMP moiety. The ESR spectrum of the spin-labeled enzyme is very similar to the spectrum of free Tempo-ATP. This suggests that the Tempo moiety of the adenylylated enzyme is slightly immobilized and possesses a rotational correlation time of less than 8×10^{-10} seconds. This rotational correlation time is at least 100-fold lower than that calculated for the entire dodecamer. This observation is in agreement with the NMR data (Proc. Natl. Acad. Sci. 75:1255, 1978) which indicates that the regulatory site is located on the surface of the enzyme. The ESR signal is quenched by the enzyme bound Mn(II) and the extent of the quenching is dependent upon the distance between the nitroxide radical and the Mn(II) binding site. The data obtained by monitoring the ESR signal as a function of Mn(II) concentration in the absence or presence of substrates such as ATP, Glu, Gln, ADP, or Pi reveal that: (i) The Mn(II) binding constant for the tight site (n_1 site) is 1.1 µM and for the catalytic site (n_2 site) is 16.7 µM, which is in reasonable agreement with the values of 1.8 µM and 18 µM determined by equilibrium dialysis for the n_1 and n_2 sites, respectively. (ii) The affinity for Mn(II) at n_2 site is enhanced significantly in the presence of ADP and Pi, or ADP and Gln, or ATP and Glu. (iii) The substrate pairs described in (ii) also induce a protein conformational change as indicated by the change in the distance between the nitroxide radical and the bound Mn(II). In addition, the ESR signal was also used to demonstrate the mutually exclusive binding between Mn(II) and Mg(II).

V. On the Mechanism of Ca(II) Activation of Calmodulin-Regulated Cyclic Nucleotide Phosphodiesterase (see Annual Report of C.Y. Huang). In this study, Ca(II) binding to calmodulin was determined using both the intrinsic tyrosine fluorescence of calmodulin or dansylated troponin C as Ca(II) indicator. The results show that the binding of Ca(II) to calmodulin exhibits both positive and negative cooperativity. In addition, a mechanistic scheme which involves all the Ca(II)-calmodulin, enzyme-calmodulin, and Ca(II)-calmodulin-enzyme complexes was proposed. Although the parameters required to describe the proposed mechanism are still under investigation, current results have revealed several important regulatory properties. They are: (i) The activated calmodulin-enzyme complex contains 4 equivalents of Ca(II); therefore, the activation of the phosphodiesterase becomes highly sensitive to the change in Ca(II) concentration. (ii) The huge increase in affinity between calmodulin and enzyme derived from Ca(II) binding can be accounted for by a relatively small decrease in the dissociation constant for each of the 4 calcium binding steps. As a consequence, Ca(II) can be dissociated relatively rapidly from the calmodulin·Ca(II)·enzyme complex; hence the enzymic activity can be modulated rapidly in response to the changes in Ca(II) concentration.

VI. Mechanism and Regulation of Rabbit Skeletal Muscle Actomyosin ATPase (for details see Annual Report of E. Eisenberg).

(a) The Lynn-Taylor model for the actomyosin ATPase (Biochemistry 10: 4617, 1971) suggests that ATP hydrolysis required the dissociation of ATP·actomyosin complex to ATP·myosin and actin. This model predicts that high actin concentration will inhibit the ATPase activity. However, using myosin subfragment-1 (S-1), we have demonstrated that (i) S-1·ATP is in rapid equilibrium with acto-S-1·ATP and that under conditions where there is a significant amount of S-1 present in acto-S-1·ATP form, there is no inhibition of the ATPase activity; (ii) At high actin concentration, the rate of ATP hydrolysis, measured either by fluorescence enhancement or by direct Pi assay, is markedly increased relative to that measured at low actin concentration. The overall results indicate that the acto-S-1 complex is not dissociated by ATP during the hydrolysis. Contrary to the Lynn-Taylor prediction, high actin concentration is not only noninhibitory to ATPase activity, it also facilitates the ATPase process to some extent.

(b) It is believed that relaxation of skeletal muscle is mediated by the troponin-tropomyosin complex which binds to F-actin. In this aspect, it has been suggested that in the presence of ATP and absence of Ca(II), tropomyosin physically blocks the binding of the myosin cross-bridge to F-actin. In this study, a stopped-flow machine was used to monitor the binding of S-1·ATP and S-1·ADP·Pi to F-actin-troponin-tropomyosin complex in the presence and absence of Ca(II). The results show that in the absence of Ca(II) under a condition when actin-activated ATPase is 96% inhibited by troponin-tropomyosin, the troponin-tropomyosin complex exerts very little effect on the binding of S-1·ATP or S-1·ADP·Pi to F-actin. Thus, the troponin-tropomyosin complex does not inhibit the actin-activated ATPase by preventing the binding of S-1·ATP or S-1·ADP·Pi to actin; instead it may inhibit the release of Pi from acto-S-1·ADP·Pi complex.

Significance to Biomedical Research

The overall objective is to gain a better understanding of how enzymes function with respect to their catalytic and regulatory properties, and to elucidate principles of interaction between effectors, regulators, and proteins.

The study of alkaline phosphatase reveals a possible regulatory role for negative cooperativity. The physical and mechanistic studies of enzymic action will enhance our knowledge of how enzymes function. This knowledge is instrumental in controlling the function of a specific enzyme by designing an effector or enzyme suicide substrate. The study on calmodulin activation of cyclic nucleotide phosphodiesterase can reveal a general mechanism for calmodulin-dependent Ca(II) regulation of enzymic activities.

Proposed Course of Research

(1) To further expand the cyclic cascade model to substrate cascade and more complex enzyme cascade systems; and to further analyze the kinetic aspect of the cyclic cascade system.

(2) To study the mechanism of the cascade reactions which regulate the state of adenylylation for glutamine synthetase. We plan to study the protein-protein interaction between P_{II} protein and ATase, UTase, and UR, and ATase-glutamine synthetase interaction, by physical, chemical, and immunological methods, to test the validity of the bicyclic cascade model, and to study the role of the effectors in this cascade system. In addition, the phosphorylation cascade involving cAMP-dependent protein kinase and phosphoprotein phosphatase will be investigated.

(3) To further explore the physical, chemical, and immunological porperties of unadenylylated and adenylylated glutamine synthetase. In particular, we shall utilize the fast reaction technique, NMR, ESR, and fluorescence polarization methods to elucidate the roles of effectors and to identify the intermediates in the catalytic cycle. Laser light scattering will be used to study protein-protein interactions.

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Bale, J.R., Huang, C.Y., and Chock, P.B.: Transient Kinetic Analysis of the Catalytic Cycle of Alkaline Phosphatase. J. Biol. Chem. 255, 1980, in press.

Wang, J.H., Sharma, R.K., Huang, C.Y., Chau, V., and Chock, P.B.: On the Mechanism of Activation of Cyclic Nucleotide Phosphodiesterase by Calmodulin. Ann. N.Y. Acad. Sci., 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00203-07 LB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cellular Regulation of Enzyme Levels

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Cynthia Oliver	Microbiologist	LB NHLBI
Other:	E. R. Stadtman	Chief,Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this investigation is to study the regulation of intracellular protein turnover in Escherichia coli and to isolate and characterize an in vitro cell-free system which catalyzes the degradation of glutamine synthetase (GS). The current studies indicate that degradation of GS by E. coli extracts might be a two-step process consisting of inactivation followed by proteolysis. The inactivation reaction was first observed in extracts of nitrogen starved Klebsiella aerogenes (Fulks, R.M. (1977) Fed. Proc. 36, 3420). Similar inactivation occurs when GS is incubated with ascorbate (Levine, R.L. (1980) Fed. Proc. 39, 401) and with catalase-free E. coli extracts. This reaction requires Fe^{3+}, NADPH, O_2 and is inhibited by catalase. Rabbit liver microsomal P_{450} mixed function oxidase system consisting of cytochrome P_{450} and NADPH cytochrome C reductase catalyzes a similar inactivation and this system has been used as a model to study inactivation without proteolysis. Techniques used in these studies have included polyacrylamide pore gradient electrophoresis, isotopic labeling, chromatographic techniques, autoradiography, enzymatic assay of functional proteins, high pressure liquid chromatography.

PHS-6040
(Rev. 10-76)

Project Description

The control of intracellular enzyme level is important in metabolic regulation. Changes in these enzyme levels reflect either changes in the rate of protein synthesis or changes in the rate of degradation. The metabolic control of the various aspects of protein synthesis has been well characterized, but little is known about intracellular degradation or its regulation. The object of this investigation is to elucidate some of the basic mechanisms underlying the process of protein degradation. The current studies have indicated that protein degradation might be a two-step process involving inactivation and proteolysis. This report is concerned primarily with the initial step, inactivation.

Major Findings

It has been exceedingly difficult to demonstrate inactivation or proteolysis of glutamine synthetase (GS) by *E. coli* cell-free extracts derived from cells grown under a variety of nutritional conditions. However, Fulks demonstrated rapid inactivation and proteolysis of GS in *K. aerogenes* during nitrogen starvation (Fulks, R.M. (1977) Fed. Proc. *39*, 3420). Subsequent studies in this laboratory revealed that the inactivation reaction required Fe^{3+}, NADPH, and O_2, and is inhibited by catalase (Fulks, R.M., Levine, R.L., and Stadtman, E.R., unpublished data). In addition, nonenzymatic treatment of GS with ascorbate produced a similar inactivation (Levine, R.L. (1980) Fed. Proc. *39*, 401). These results suggested that degradation of GS might be at least a two-step process consisting of an initial inactivation followed by proteolysis. It was possible that the inability to detect proteolysis of GS by *E. coli* extracts was due to the presence of high catalase levels which inhibited the initial inactivation. When similarly prepared extracts of *K. aerogenes* and *E. coli* were tested for catalase levels, it was found that *E. coli* extracts possessed high catalase levels, while *K. aerogenes* extracts were nearly devoid of catalase activity. Extract mixing experiments revealed that *E. coli* extracts inhibited the inactivation reaction in *K. aerogenes* extracts.

These results suggested that *E. coli* extracts lacking catalase activity might possess the capacity to degrade GS. The first approach was a genetic one. A catalase-deficient mutant was obtained using a selection based on a requirement for δ-aminolevulinic acid, an intermediate in heme synthesis. Another, ferrochelatase mutant, was obtained from the *E. coli* genetic stock maintained by Dr. Barbara Bachmann. Each of these mutants possessed a different lesion in heme synthesis and neither was a catalase apoprotein mutant. When extracts of these mutants were prepared and tested for the capacity to degrade GS and other proteins, only azocasein, azoalbumin, and denatured hemoglobulin were proteolyzed. Except for one experiment which could not be repeated, GS was degraded only slowly and only after prolonged incubations. An explanation for these observations was not readily apparent. It must be emphasized, however, that heme mutations are not specific for catalase and presumably affect all heme proteins and therefore a variety of metabolic processes.

An alternative approach was to obtain catalase-free *E. coli* extracts by purification and to test these extracts for the capacity to degrade GS. Catalase-free preparations were obtained by treatment of crude extracts with streptomycin sulfate, followed by mercaptoethanol precipitation, ammonium sulfate precipitation, and then chromatography on DEAE cellulose and sepharyl S-300. When the resulting preparations were incubated with GS in the presence of Fe^{3+}, NADPH, O_2, and ATP rapid proteolysis was observed. Ascorbate-inactivated GS was degraded more readily than native GS. Degradation of native GS is inhibited by catalase, whereas catalase has no effect on the degradation of ascorbate-inactivated GS. These same preparations catalyze inactivation of GS.

In the course of these studies, a new rapid method for quantitating proteolysis was developed which involved SDS high pressure liquid chromatography of timed samples on an SW3000 size exclusion column. Under appropriate conditions, it was possible to quantitate loss of GS subunit protein as a function of time by peak area integration. Using this method, it was found that large fragments (17,000 and 35,000) observed in subtilisin model studies (Dautry-Varsat, A., Cohen, G.N., and Stadtman, E.R. (1979) J. Biol. Chem. 254, 3124), if produced, do not accumulate, and there is rapid transient generation of multiple small molecular weight fragments of 10,000 or less.

In order to study the inactivation reaction without proteolysis, it was necessary to either separate the respective activities, inhibit the proteolysis, or find a purified enzyme system capable of carrying out inactivation. Since the inactivation reaction requirements were similar to P_{450}-catalyzed mixed function oxidase reactions, the latter alternative was selected. The P_{450} components were obtained from several investigators (Dr. Minor Coon, University of Michigan, Dr. Harry V. Gelboin, NIH, and Dr. Paul Thomas, Hoffman LaRoche, Inc.) and tested for the capacity to inactivate GS. It was found that the microsomal P_{450} system catalyzed an NADPH-dependent inactivation of GS that was similar to the inactivation observed with the bacterial extracts and with ascorbate. The reaction required Fe^{3+}, O_2, and was inhibited by catalase. The reaction is stimulated by ATP and ADP, but not by CTP, GTP, UTP, AMP, or the ATP analogue AMP-PNP. The nature of the nucleotide stimulation is not known. In addition, unadenylylated GS is protected from inactivation by ATP and glutamate, whereas the adenylylated GS is unaffected by these cosubstrates. Neither glutamate or ATP alone has a protective effect. These results suggest that the inactivation reaction may be physiologically important.

It was then found that catalase-free E. coli preparations which were capable of GS inactivation and proteolysis possessed both P_{450} and NADPH-cytochrome C reductase activity. However, definitive proof that these components function directly in the GS inactivation reaction in E. coli extracts awaits purification and reconstitution of the respective activities. These catalase-free preparations also proteolyze microsomal P_{450} inactivated GS more readily than native GS.

Proposed Course of Action

We have partially characterized a reaction which inactivates E. coli GS and renders the GS more susceptible to proteolysis. This reaction is catalyzed enzymatically by a purified rabbit liver microsomal P_{450} mixed function oxidase system. The inactivation requires NADPH, O_2, and Fe^{2+}, and is observed in Klebsiella extracts and E. coli catalase-free extracts. It is important to determine if this inactivation is a general property of mixed function oxidation systems, and therefore if Pseudomonas pudita purified P_{450} cam system also catalyzes this reaction. It is also important to determine the nature of the inactivation reaction. Dr. R. L. Levine has obtained evidence suggesting that a single tyrosine residue per GS subunit is altered during asocorbate inactivation, and this modification leads to characteristic UV spectral changes. If the enzymatic inactivation leads to a similar modification which exhibits similar spectral changes, it may be possible to monitor and quantitate this reaction spectrally. It would then be possible to assay a P_{450}-directed modification in proteins where one or more susceptible residues are not active site-associated residues, or do not affect enzyme activity. The potential difficulty with these studies is quantitating small UV spectral changes in the presence of NADP-NADPH. However, it has been found that P_{450} is capable of oxidizing certain substrates by a peroxidase-type reaction in the presence of peroxy compounds (Blake, R.C. and Coon, M.J. (1980) J. Biol. Chem. 255, 4100). If the inactivation reaction occurs in the presence of organic peroxides, and if this reaction bypasses the NADPH coupling system, it might be possible to monitor the reaction spectrally in the absence of NADP-NADPH. Other experiments designed to identify the target residue or residues might involve the use of $K^{125}I$. If inactivation occurs in the presence of $K^{125}I$, and if labeled iodide is incorporated into the inactivated product, then labeled products could be recovered following hydrolysis and amino acid analysis.

It would be important to purify and reconstitute the component or components necessary for the inactivation reaction in E. coli. In addition, if labeled native GS was prepared, enzymatically inactivated and reisolated, this material could be used as a substrate to assay and therefore isolate a protease or proteases which degrade the modified or inactivated protein.

There are many questions concerning the regulation of inactivation. In Klebsiella extracts, in E. coli, and in the microsomal P_{450} system, catalase inhibits the inactivation reaction. Catalase is found in all cells from which the inactivating activities were derived. If this inactivating reaction functions physiologically, then the catalase effect must be explained. Several possibilities have been suggested, for example, catalase activity might be highly regulated in vivo, catalase activity and the inactivating activity might be physically separated by compartmentation or the in vivo inactivation reaction proceeds via an organic peroxide which is resistant to catalase activity. There is much to be learned about this inactivation reaction, the nature of P_{450}-mixed function oxidase-directed enzyme modification or inactivation, its physiological function and potential role in intracellular protein turnover.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00204-13 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Protein Structure: Enzyme Action and Control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ann Ginsburg	Chief, Section on Protein Chemistry	LB NHLBI
Others:	Andrew Shrake	Staff Fellow (until 9/30/80)	LB NHLBI
	Edward J. Whitley, Jr.	Staff Fellow (until 8/29/80)	LB NHLBI
	Eileen G. Gorman	Staff Fellow (10/9/79 -)	LB NHLBI
	Michael R. Maurizi	Staff Fellow (6/16/80 -)	LB NHLBI
	Paula M. Grant	Q-authority appointment (under 1040 hours)	LB NHLBI

Cooperating Units

J.B. Hunt, Associate Professor, Department of Chemistry, The Catholic University of America

Howard K. Schachman, Professor of Molecular Biology and Biochemistry, University of California, Berkeley, California 94720

F.C. Wedler, Associate Professor Biochemistry, Penn State University, University Park, Pennsylvania 16802

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Protein Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 5.0	PROFESSIONAL: 4.5	OTHER: 0.5

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SUMMARY OF WORK (200 words or less - underline keywords)

Research in this Section consists of studies on the physical and chemical properties of proteins of biological interest and the roles of ligand binding and protein-protein interactions in enzyme catalysis and regulation.

(1) Interactions of divalent cations, substrates, and inhibitors with glutamine synthetase from Escherichia coli have been studied by calorimetry, ultracentrifugation, equilibrium dialysis, pH, spectral, thermal perturbation, and kinetic techniques. L-Methionine-SR-sulfoximine (a transition state analog) promotes both local and gross conformational differences between unadenylylated and adenylylated enzymes; thermodynamic parameters for the binding of the S and R diastereoisomers of this analog differ.

The unadenylylated Mn-enzyme catalyzes a novel, AMP-dependent (reversible) synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine.

(2) A calorimetric estimate of the enthalpy change for the substrate-promoted conformational transition of aspartate transcarbamoylase from E. coli is -6 kcal/mol. From heats of subunit assembly in the presence and absence of a bisubstrate analog, the substrate-linked subunit interaction enthalpy change is + 22 kcal/mol.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

(1) To study the physical and chemical properties of glutamine synthetase from *Escherichia coli*, particularly with respect to the correlation of the structure and catalytic function of this enzyme.

(2) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation.

(3) To investigate the role of AMP in supporting γ-glutamyl transfer and other activities of unadenylylated manganese-glutamine synthetase.

(4) Kinetic and equilibrium studies of protein-metal ion interactions, using glutamine synthetase and dye-metal ion complexes, using the enzyme + Me^{2+} in the presence of pH-indicator dyes, and using the manganese-enzyme and pyridine-2,6-dicarboxylic acid in the absence and presence of a pH indicator dye.

(5) Ultracentrifugal, calorimetric, and electrophoretic studies to determine macromolecular properties of biologically important proteins.

(6) To measure the enthalpy change for the substrate-promoted transition of aspartate transcarbamoylase from *E. coli*, using two calorimetric approaches and ultracentrifugal techniques.

Major Findings

(1) Interactions of *L*-methionine sulfoximine and substrates with unadenylylated and adenylylated glutamine synthetase forms from *E. coli*. (Investigators: A. Shrake, E.G. Gorman, and A. Ginsburg). Glutamine synthetase, a strictly regulated enzyme in *E. coli*, is a dodecamer with twelve catalytic sites. It was previously reported that the binding of *L*-methionine-*SR*-sulfoximine (a transition state analog) at the subunit catalytic site promotes different local and different gross structural changes in unadenylylated (GS) and adenylylated ($GS_{\overline{12}}$) glutamine synthetase. Negative cooperativity (with Hill coefficients, $n_H < 1$) in binding this analog to the enzyme was observed also.

The present studies were undertaken to characterize the interaction of glutamine synthetase with the S and R diastereoisomers of *L*-methionine sulfoximine, when added separately or as a 1:1 mixture of the S- and R-isomers. For these studies, the separate S- and R-isomers of *L*-methionine sulfoximine were provided by Dr. F.C. Wedler (Penn State University). Table I summarizes results (obtained by A. Shrake) from UV spectrophotometric titrations of $MnGS_{\overline{0}}$, $Mn{\cdot}ADP{\cdot}GS_{\overline{0}}$, and $MnGS_{\overline{12}}$ complexes with L-methionine-*S*-sulfoximine, *L*-methionine-*R*-sulfoximine, and a 1:1 mixture of the S- and R-isomers, using ligand-promoted protein difference spectra. The 1:1 mixture of diasteroisomers gave the same values (within experimental error) as obtained before with commercial *L*-methionine-*SR*-sulfoximine (Shrake *et al.*, 1980). Although only S isomer is in the correct configuration to be phosphorylated by ATP on the enzyme surface, both the S and the R isomers produce the same protein difference spectrum for each enzyme species studied. However, the half-saturation values $[S]_{0.5}$ for the R isomer were consistently greater than those for the S isomer. This suggests that subunit interactions play a role in binding, since the unadenylylated, manganese enzyme has about the same affinity for the two isomers in the commercial mixture of S and R isomers (Shrake et al., 1980). That is, when ~ 50% of the sites of the dodecamer are filled with S isomer, the unoccupied remaining sites must have a greater affinity for the R isomer than when only R isomer is present. Experiments are in progress to demonstrate that this type of subunit interaction occurs. With only the S isomer present, negative cooperativity in binding this analog is observed with the unadenylylated and adenylylated Mn-enzymes.

Table I. Spectrophotometric Titration Data: Glutamine Synthetase + L-Methionine Sulfoximine (S and R Diastereoisomers)

Binding reaction of GS subunit[a]	Peak (nm)	Trough (nm)	$\Delta\ \varepsilon_{max}$ (M subunit)$^{-1}$	$[S]_{0.5}$ mM	n_H
$\text{Mn}\cdot\text{GS}_{\overline{0}}$ + S isomer	285.5	281.5	520	0.031	0.8
+ R isomer	"	"	450	0.39	0.9
+ (S + R)	"	"	544	0.062	0.8
$\text{Mn}\cdot\text{ADP}\cdot\text{GS}_{\overline{0}}$ + S isomer	285.1	281.6	398	< 0.001[b]	N.D.
+ R isomer	"	"	440	0.063	0.6
+ (S + R)	"	"	434	~ 0.010	1.0
$\text{Mn}\cdot\text{GS}_{\overline{12}}$ + S isomer	284.0	297.0	740	0.11	0.5
+ R isomer	"	"	655	0.91	1.0
+ (S + R)	"	"	700	0.15	0.7

[a] All measurements were made at 25°C in 20 mM Hepes/KOH -100 mM KCl-1.0 mM $MnCl_2$ buffer at pH 7.1; $GS_{\overline{0}}$ and $GS_{\overline{12}}$ are the unadenylylated and adenlylylated enzyme complexes, respectively. For forming the Mn·ADP·GS complex, a free concentration of 0.1 mM ADP was used.

[b] The affinity of this complex for the S isomer is too great to measure a $[S]_{0.5}$ value by this technique.

Concurrent calorimetric studies of E.G. Gorman have provided ΔH values and proton uptake and release data for the interactions of the unadenylylated Mn enzyme with S, R, and a 1:1 mixture of S and R isomers of L-methionine sulfoximine. Thermal titrations for each ligand and for the mixture of isomers were performed in two buffers (Hepes/KOH and Tris/HCl, at pH 7.1, 30°C) which do not interact with the protein and have different heats of protonation; $\Delta(\Delta H)$ = 6.2 kcal/mol for the difference in proton uptake between these two buffers. These data give information on the number of protons involved in each binding reaction and on cooperative effects that may be manifested by heats of binding in different ranges of ligand saturation and allow correction of observed heats to enthalpies of binding. Combining the calorimetric data with $\Delta G'$ values (calculated from $[S]_{0.5}$ values of Table I or from thermal titration curves) gives the thermodynamic parameters summarized in Table II.

Table II. Thermodynamic Parameters for the Interaction of Unadenylylated, Manganese Glutamine Synthetase with L-Methionine Sulfoximine (S and R Diastereoisomers) or with L-Glutamine

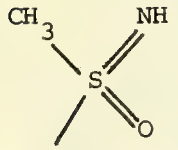
Binding reaction of GS subunit[a]	$\Delta G'$ kcal (mol subunit)$^{-1}$	ΔH kcal (mol subunit)$^{-1}$	ΔS cal (deg·mol)$^{-1}$	Proton Effect Eq (mol subunit)$^{-1}$
Mn_2GS + S isomer	- 6.2	- 19.0	- 42	0.7 H^+ uptake
+ R isomer	- 4.9	- 9.6	- 16	0.3 H^+ release
+ (S + R)	- 6.0	~ - 23	~ - 56	~ 1.5 H^+ uptake
+ L-glutamine[b]	- 3.0	- 9.7	- 22	0

[a] Experiments with GS_I^- at 30°C in 20 mM Hepes/KOH or 100 mM Tris/HCl buffers at pH 7.1 containing 100 mM KCl and 1.0 mM $MnCl_2$.

[b] These data are from A. Shrake, D.M. Powers, and A. Ginsburg (1977) Biochemistry 16, 4372-4381 and are included for comparison.

The data in Table II show interesting differences between the modes of binding the S and R diastereoisomers of L-methionine sulfoximine to glutamine synthetase. This type of information is quite unique and potentially can be used for mapping interactions between substrates and the active site of the glutamine synthetase subunit.

When bound to the enzyme, the S isomer is believed to assume a conformation in which the nearly tetrahedral geometry of the sulfoximine moiety is closely analogous to that of the postulated tetrahedral L-glutamate intermediate formed during the course of L-glutamine synthesis. As a transition state analog proposed by A. Meister, the S-methyl group of L-methionine-S-sulfoximine occupies the ammonia site of the enzyme and the sulfoximine nitrogen atom is positioned for phosphorylation by ATP. The relative positions of the nitrogen and oxygen atoms are reversed in the R isomer. The heat for binding the S isomer to the Mn_2-subunit complex is about twice as negative as that for binding the R isomer or that for binding L-glutamine (Table II). The ΔS data indicate that the binding of the S isomer has a greater ordering effect than that produced by R isomer or by L-glutamine binding. Furthermore, the binding of the S isomer and R isomer to the enzyme subunit produces a 0.7 H^+ uptake and a 0.3 H^+ release, respectively. For these binding reactions, Hill coefficients from calorimetric data were essentially the same as those given in Table I, indicating about the same cooperativity in heats as in spectral perturbations. The preliminary data with the mixture of S and R isomers suggest that the



heat of binding the R isomer becomes more negative in the presence of S isomer, since the binding of S isomer apparently increases the affinity of other subunits for the R isomer. The proton uptake in this case may partially reflect conformational changes arising from subunit interactions.

We plan to continue calorimetric studies, using nucleotides and nucleotide analogs, Mg^{2+} substituted for Mn^{2+}, and also L-methionine-S-sulfoximine phosphate for binding experiments with the unadenylylated enzyme. In addition, comparable data to those given in Table II will be obtained with the fully adenylylated, manganese enzyme.

In view of the protein difference spectra produced by the binding of L-methionine sulfoximine to glutamine synthetase (Table I), we carefully looked for similar conformational changes produced by the binding of L-glutamate and L-glutamine to Mn-enzyme forms. In general, we found small but real spectral perturbations of glutamine synthetase in the presence of high concentrations of these substrates using the 0.02 absorbance scale of a Cary Model 219 spectrophotometer. With unadenylylated glutamine synthetase ($GS_{\bar{1}}$) and 60 mM L-glutamate, a small tyrosyl perturbation was observed with the amplitude at 283 nm equal to about 20% of that produced by saturating L-methionine sulfoximine. With the fully adenylylated enzyme ($MnGS_{\overline{12}}$), the difference spectrum produced by binding L-glutamine was substantial (66% of the amplitude observed with saturating L-methionine-SR-sulfoximine), and, as in the case of the analog, indicated that covalently bound AMP groups were perturbed by L-glutamine binding. A spectrophotometric titration of $MnGS_{\overline{12}}$ with L-glutamine gave $K_D^{Gln} \simeq 5$ mM, which is the same value as determined previously by calorimetry. Spectral perturbations produced by L-glutamate binding to $MnGS_{\overline{12}}$ were smaller and different from those produced by L-glutamine. The feasibility of continuing to measure spectral perturbations produced by substrate binding to unadenylylated and adenylylated glutamine synthetase will depend on the capability of a UV spectrophotometer such as the Hewlett-Packard Model 8450.

(2) Studies of a reversible thermal transition in glutamine synthetase. (Investigator: A. Shrake). As previously reported, a partial unfolding (without subunit dissociation) of glutamine synthetase can be observed by temperature-induced protein difference spectra corresponding to the exposure of tryptophan and tyrosine side chains. We have attempted to demonstrate that the spectral melting curves (measured under equilibrium conditions), which first indicated the presence of the reversible transition, are paralleled by changes in catalytic activity in higher temperature regions. An Arrhenius plot of Mn-catalyzed γ-glutamyl transfer activity at pH 7.0 with unadenylylated enzyme was linear from 4°C to ~ 55°C. However, above 55°C, the plot was concave downward with measured activities less than those predicted from a linearly extrapolated Arrhenius plot. At 72°C, the measured activity was 50% of that predicted. The loss in activity is reversible. Except for L-glutamine and hydroxylamine, all substrates were saturating at 70°C (at 37°C, all substrates are saturating). The K_m's for L-glutamine and hydroxylamine were determined at 72°C and correction of the measured activity for subsaturation with these substrates still gave a saturating activity that was only 80% of that predicted by the extrapolated Arrhenius plot. Thus, part of the

reversible loss of catalytic activity from 55-72°C is probably due to the thermal transition. This is further indicated by a van't Hoff plot of the K_m's for L-glutamine at 15, 30, 45, and 72°C. The van't Hoff plot is linear for the lowest three temperatures with an associated van't Hoff ΔH^o of -1 kcal/mole of subunit [to be compared to $\Delta H = -7.4$ kcal/mol measured for the binding of L-glutamine to the Mn·GS·ADP complex at 30°C, pH 7.1; Shrake, A., Powers, D.M., and Ginsburg, A., Biochemistry 16, 4372-4381, 1977]. The extrapolated van't Hoff plot predicts a value of 3.9 mM for K_m^{gln} at 72°C, whereas the measured value is 28 mM. The data suggest a reversible thermal transition in the enzyme occurs such that the high temperature (partially unfolded) molecular form has a decreased V_{max} and an increased K_m^{gln} relative to the folded, native protein.

(3) Studies of a novel reaction catalyzed by E. coli glutamine synthetase: AMP-dependent synthesis of pyrophosphate and L-glutamate from orthophosphate and L-glutamine. (Investigators: E. J. Whitley, Jr., and A. Ginsburg). AMP (substituted for the nonconsumable substrate ADP) supports the γ-glutamyl transfer reaction:

$$\underline{L}\text{-Glutamine} + NH_2OH \xrightarrow[(AMP)]{(Mn^{2+},\ P_i)} \gamma\text{-Glutamylhydroxamate} + NH_4^+ \tag{1}$$

catalyzed by unadenylylated glutamine synthetase from E. coli (J.B. Hunt, P.Z. Smyrniotis, A. Ginsburg, and E.R. Stadtman, Arch. Biochem. Biophys. 166, 102-124, 1975). In current studies of this reaction, it was discovered that AMP supports the reversible synthesis of PP_i from P_i in the following reaction:

$$\underline{L}\text{-Glutamine} + 2\ P_i \overset{(Mn^{2+},\ AMP)}{\rightleftharpoons} \underline{L}\text{-Glutamate} + PP_i + NH_4^+ \tag{2}$$

catalyzed by the unadenylylated enzyme ($GS_{\overline{1}}$). In reactions 1 and 2, Mg^{2+} will not substitute for Mn^{2+}, and adenylylated glutamine synthetase ($GS_{\overline{12}}$) is inactive. However, reaction 2 is related to the reverse biosynthetic reaction:

$$\underline{L}\text{-Glutamine} + ADP + P_i \xrightarrow[(\text{or Mn-}GS_{\overline{12}})]{(\text{Mg- or Mn-}GS_{\overline{0}})} \underline{L}\text{-Glutamate} + ATP + NH_4^+ \tag{3}$$

which is catalyzed by both the unadenylylated Mg- or Mn-enzyme and the adenylylated Mn-enzyme (M.D. Denton and A. Ginsburg, Biochemistry 9, 617-632, 1970). In order to identifiy products and to further characterize these reactions, reactions 2 and 3 were run at pH 7.2 and 30°C in the presence of $MnCl_2$ (or $MgCl_2$) and L-glutamine with ADP + $^{32}P_i$, AMP + $^{32}P_i$, or [^{32}P]AMP + P_i. Products were identified by charcoal adsorption, thin layer chromatography on PEI-cellulose with autoradiography, and chromatography on Dowex-1 (Cl^--form) with scintillation counting of effluent fractions.

In reaction 2, it has been found that L-glutamine is required for PP_i synthesis; the hydrolysis of the L-glutamine amide bond is coupled to the stoichiometric synthesis of pyrophosphate, although as PP_i accumulates, additional hydrolysis of L-glutamine occurs in a secondary reaction catalyzed by the [manganese·enzyme·AMP·PP_i] complex. The synthesis of PP_i probably occurs at the subunit catalytic site in the positions normally occupied by the β,γ phosphates of ATP. To promote PP_i synthesis, AMP apparently binds to the subunit catalytic site, rather than to the allosteric inhibitor site; equilibrium binding results suggest that P_i directs the binding of AMP to the enzyme subunit. Pyrophosphate is synthesized by the unadenylylated, manganese enzyme at ~ 2% of the rate of that of ATP in the reverse biosynthetic reaction. If P_i is replaced by arsenate, the enzymatic rate of the AMP-supported hydrolysis of L-glutamine is 100-fold faster than is PP_i synthesis, and is one-half the rate of the ADP-supported, irreversible arsenolysis of L-glutamine:

$$\text{L-Glutamine} + H_2O \xrightarrow[\text{Arsenate}]{Me^{2+}\text{, ADP or AMP}} \text{L-Glutamate} + NH_3 \qquad (4)$$

Arsenolysis also is supported by GMP and IMP, suggesting that the catalytic site of glutamine synthetase has a rather broad specificity for the nucleotide base.

The reactions supported by AMP directly relate to the mechanism of glutamine synthetase catalysis in which the nucleotide site of the enzyme subunit must be occupied either by ADP or by AMP for synthesis of the β,γ phosphate bond to occur. Table III summarizes activity data for unadenylylated glutamine synthetase so that the activities in AMP- and ADP-supported reactions with either Mn^{2+} or Mg^{2+} present may be compared.

Table III. Unadenylylated Glutamine Synthetase Activity

Activity data are expressed for initial rates of nmoles product formed at pH 7.2 per minute at 30°C per mg $GS_{\bar{1}}$ in 50 mM Hepes-KOH, 100 mM KCl (± 40 mM NH_2OH), and either 12 mM AMP or 5 mM ADP with 0.5 mM $MnCl_2$ or 50 mM $MgCl_2$. Reaction mixtures (except for that of -2) also contained 15 mM L-glutamine and 10-13 mM P_i or arsenate. For the reverse of reaction 2, 200 mM L-glutamate, 200 mM NH_4Cl, and 1 mM PP_i were used.

Reaction (in text)	Products measured	Nucleotide and divalent cation support: AMP Mn^{2+}	AMP Mg^{2+}	ADP Mn^{2+}	ADP Mg^{2+}
2	Glu, PP_i	1.8	0		
-2 (reverse 2)	P_i	0.65	0		
-3 (reverse 3)	Glu, ATP			120	480
4 (arsenolysis)	Glu	200	0.7	410	46
1 (with P_i)	γ-glu·NHOH[a]	2500	0	5000	6000
1 (with arsenate)	γ-glu·NHOH[a]	24,000	0	41,000	7000

[a] γ-glu·NHOH, γ-glutamylhydroxamate.

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(4) Glutamine synthetase-Mn^{2+} interactions (Investigators: J.B. Hunt, P.M. Grant, and A. Ginsburg). Xylenol orange (a metal ion binding dye) has been used by J.B. Hunt in equilibrium and kinetic studies of Mn^{2+} interactions with the structural n_1 sites of unadenylylated glutamine synthetase from E. coli. The following properties of xylenol orange (determined in these studies) make it a useful reagent for studying protein-Mn^{2+} interactions: (a) Formation of a 1:1 complex between xylenol orange and Mn^{2+} at pH 7.2 (25°C) with $K_A' = 1.6 \pm 0.1 \times 10^5\ M^{-1}$ produces a considerable increase in the molar extinction coefficient ($\Delta \varepsilon = 1.9 \times 10^4\ M^{-1}\ cm^{-1}$ at 585 nm); (b) Xylenol orange does not bind to glutamine synthetase; (c) Stop-flow measurements at pH 7.2 and 15°C of xylenol orange interaction with Mn^{2+} give a second order rate constant of $4.1 \times 10^6\ M^{-1}\ sec^{-1}$ for complex formation, and a first order rate constant of 29 sec^{-1} for Mn^{2+} dissociation from the dye. Xylenol orange was used spectrophotometrically at 25°C (pH 7.2) to measure a time-dependent increase in the glutamine synthetase binding constant for Mn^{2+} that occurs during the Mn^{2+}-promoted conformational change (with a half-time of 110 seconds at 25°C). The increase from $K_A' = 1.5 \times 10^5$ to $1.9 \times 10^6\ M^{-1}$ gives an estimate of $\Delta(\Delta G') = +18$ kcal (mol enzyme)$^{-1}$ for the protein conformational change at 25°C (pH 7.2). In stop-flow experiments, xylenol orange was used to measure the off-rate of Mn^{2+} from n_1 sites of the enzyme (after completion of the Mn^{2+}-promoted conformational change); Mn^{2+} dissociation was first order with a half-time of 0.22 seconds at 15°C. These studies also utilized pyridine-2,6-dicarboxylic acid (dipicolinic acid) which forms a 2:1 complex with Mn^{2+} with a small absorption increase at 290 nm and binds Mn^{2+} without proton release so that it can be coupled to a pH indicator dye. Experiments with dipicolinate in the absence and presence of bromothymol blue verified the off-rate of Mn^{2+} (coincident with a fast proton uptake) from glutamine synthetase and, furthermore, showed that the slow proton uptake after the removal of Mn^{2+} from the enzyme (which is synchronous with the protein conformational change) is the result of a pK_a change through perturbation of an ionizable amino acid side chain not directly involved in Mn^{2+} binding. A scheme for Mn^{2+} binding to n_1 sites of glutamine synthetase in two states (a low- and a high-affinity conformation) fits these data.

The rate of proton release accompanying Mn^{2+} binding to n_1 and n_2 metal ion sites of glutamine synthetase in a preliminary stop-flow experiment using 30 μM subunits and 60 μM Mn^{2+} in the presence of bromothymol blue (~ 30 μM) was measured. The proton release was second order with a rate constant of ~ 178 $sec^{-1}\ M^{-1}$ at 15°C. However, there appears to be an interaction between the enzyme and bromothymol blue that requires investigation. From other studies, an upper limit of 0.05 second is set for the half-time of Mn^{2+} dissociation from n_2 sites at 15°C.

We also are investigating the use of fluorescein complexone (a fluorescent metal ion dye) in studies of protein-Mn interactions. In preliminary studies, commercial preparations of fluorescein complexone were impure. P. Grant has synthesized fluorescein complexone (FC) from fluorescein and currently is characterizing the product. The NMR and elemental analyses were good. In fluorescence measurements, the maximum emission is at 520 nm with excitation at 472 nm. Mn^{2+} binding to FC produces a 90-95% quench of the fluorescent signal and titrations of FC with Mn^{2+} will give K_A' values. The absorption maximum is at 492 nm with $\varepsilon_M = 63,500\ M^{-1}\ cm^{-1}$; Mn^{2+} decreases the absorbance at 492 nm by 8% with $\Delta\ \varepsilon_M = 5 \times 10^3\ M^{-1}\ cm^{-1}$. The stoichiometry of the dye-Mn complex, the affinity constant of FC for Mn^{2+}, the temperature dependence of K_A', and the on and off rates for Mn^{2+} interaction with FC have yet to be established.

(5) Calorimetric estimate of the enthalpy change for the substrate-promoted conformational transition of aspartate transcarbamoylase from E. coli. (Investigators: A. Shrake, A. Ginsburg, and H.K. Schachman, Fogarty Scholar in Residence at NIH, 1977-78). These studies are described in detail in last year's annual report (Project No. Z01 HL 00204-12 LB, 1978-79). The study sets an important precedent for obtaining the enthalpy change for a ligand-promoted conformational transition of a protein. In addition, the change in subunit interaction enthalpy promoted by the binding of a ligand to an oligomeric protein is obtained. Data analyses and the preparation of a manuscript describing this work will be completed this year.

To summarize briefly, we used two calorimetric approaches and a high-affinity bisubstrate analog N-(phosphonacetyl)-L-aspartate (PALA) for binding to aspartate transcarbamoylase (ATCase) to promote the conformational transition of the enzyme. In method (a) $\Delta(\Delta\ H)$ measurements of PALA binding to ATCase at different stages of the conformational transition (monitored by the difference sedimentation technique) in $K\text{-}PO_4$ and $K\text{-}PO_4$/TES buffers at pH 7.0 allow calculation of the heat of the conformational transition from an initial T state to a final R state ($\Delta H_{T\to R}$), the intrinsic heat of binding PALA (ΔH_{PALA}), and a measure of proton uptake or release associated with the conformational change and the binding of PALA. [For example, $\Delta H'$ values for binding the first two and the last two equivalents of PALA to the enzyme can be represented by two simultaneous equations:

$\Delta H_{0\to 2} = 1/3\ \Delta H_{PALA} + 1/2\ \Delta H_{T\to R}$ and $\Delta H_{4\to 6} = 1/3\ \Delta H_{PALA} + 0\ \Delta H_{T\to R}$ which can be solved for $\Delta H_{T\to R}$ and for ΔH_{PALA}.] In method (b) the heats of assembly of catalytic trimers (C) and regulatory dimers (R) into ATCase (C_2R_3) in the absence and presence of PALA, the heat of binding PALA to the isolated catalytic subunit (C), and the value of ΔH_{PALA} from method (a) gives another estimate of $\Delta H_{T\to R}$ and also the PALA-linked subunit interaction enthalpy change. The two approaches in methods (a) and (b) give: $\Delta H_{T\to R} = -6 \pm 3$ and -14 ± 9 kcal/mol of ATCase, respectively, at pH 7.0 and 30°C; a proton uptake of 2 to 6 equivalents of H^+/mol of ATCase accompanies the protein conformational change. With $\Delta G'_{T\to R} = +\ 3.3$ kcal/mol of ATCase (Howlett, G.J., Blackburn, M.N., Compton, J.G., and Schachman, H.K., Biochemistry 16, 5091-5099, 1977) and the best estimate of $\Delta H_{T\to R}$ from method (a), $\Delta S_{T\to R} = -31$ cal $(\text{deg}\cdot\text{mol of ATCase})^{-1}$ and $-T\Delta S = +\ 9$ kcal/mol. Thus, the T→R transition of ATCase is entropically controlled at 30°C. Sedimentation analyses of assembly reactions were consistent with the binding of PALA to C subunits causing a weakening of regulatory and catalytic interchain contacts. From the heats of subunit assembly in the presence and absence of PALA, the PALA-linked subunit interaction enthalpy change is $+\ 22 \pm 8$ kcal/mol of ATCase, indicating that the corresponding entropy change is positive ($\lesssim +\ 80$ cal/deg·mol).

Significance to Biomedical Research

The regulation and control of enzymic activities in vivo is of fundamental importance in cellular metabolism. Through studies in vitro, these processes can be understood more fully. The studies of structural changes that can be induced in a protein macromolecule are important in understanding cellular processes on a molecular basis.

Proposed Course of Research

(1) To study conformational and stabilization changes of a protein macromolecule effected through the specific binding of small molecules and the relationship of such effects to enzyme catalysis and regulation. Ultracentrifugation, microcalorimetry, spectral, viscometry, fluorescence, equilibrium binding, pH, electrophoretic, and kinetic techniques will be used.

(2) To study mutual interactions of divalent cations, substrates (or substrate analogs), and inhibitors with glutamine synthetase from E. coli. Covalent modification will be used to obtain information on the topography of divalent cation, substrate, and inhibitor sites. The interactions between glutamine synthetase and the resolved S- and R-isomers of L-methionine-SR-sulfoximine will be investigated by calorimetry and other physical chemical techniques. Protein conformational changes produced by the binding of substrates and substrate analogs to glutamine synthetase will be investigated further.

(3) To characterize the thermal transition of glutamine synthetase more fully in terms of kinetic parameters. Also, the nature of the temperature-induced unfolding reaction (local vs temperature-induced macromolecular shape changes) will be investigated by viscometry, ultracentrifugation, and CD techniques.

(4) Kinetic and equilibrium studies of the interactions between metal ions and glutamine synthetase will be continued. For this purpose, metal ion binding dyes, pyridine-2,6-dicarboxylic acid and pH indicator dyes will be used.

(5) Glutamine synthetase from brain will be purified in order to determine the role of divalent cations in the catalytic mechanism of the mammalian enzyme.

(6) The work describing our methods of estimating the heat for the substrate-promoted conformational transition of aspartate transcarbamoylase (ATCase) from E. coli (in collaboration with H.K. Schachman, University of California, Berkeley, California) will be submitted for publication. For additional information on the thermodynamics of assembly and ligand-promoted confomrational transitions, ATCase (C_2R_3) also will be assembled from regulatory dimer (R) and an incomplete ATCase molecule (C_2R_2) lacking two regulatory chains. The incomplete C_2R_2 will be prepared in the laboratory of H.K. Schachman where procedures for preparing large quantities of C_2R_2 are being developed.

Publications

Shrake, A., Whitley, E.J., Jr., and Ginsburg, A.: Conformational Differences between Unadenylylated and Adenylylated Glutamine Synthetase from *Escherichia coli* on Binding *L*-Methionine Sulfoximine. J. Biol. Chem. *255*: 581-589, 1980.

Hunt, J.B., and Ginsburg, A.: Mn^{2+} and Substrate Interactions with Glutamine Synthetase from *Escherichia coli*. J. Biol. Chem. *255*: 590-594, 1980

Whitley, E.J., Jr., and Ginsburg, A.: A Novel Reaction Catalyzed by Unadenylylated Glutamine Synthetase from *Escherichia coli*: AMP-Dependent Synthesis of Pyrophosphate and *L*-Glutamate from Orthophosphate and *L*-Glutamine. J. Biol. Chem., 1980 (in press).

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P.I.:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI
	Belinda Seto	Staff Fellow (terminated 3/4/80).	
	Gregory Dilworth	Staff Fellow (see indiv. report)	LB NHLBI
	Sue H. Neece	Part time Employee (see indiv. report)	LB NHLBI
	Maris Hartmanis	Visiting Investigator (Swedish support) (see invidivual report)	LB NHLBI
	Wei-Mei Ching	Staff Fellow (started 1/27/80) (see individual report)	LB NHLBI
	Arthur Wittwer	Staff Fellow (started 5/5/80).	LB NHLBI

COOPERATING UNITS (if any)

None

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Five of the presently known six selenoenzymes are produced by bacteria and four of these are under investigation in this laboratory. These are selenoprotein A of glycine reductase, formate dehydrogenase of Methanococcus vannielii, nicotinic acid hydroxylase of Clostridium barkeri and thiolase of Clostridium kluyveri. In addition studies are underway on the identification and mode of formation of seleno-tRNAs (modified with Se in the polynucleotide portion of the molecule) which were first discovered in C. sticklandii. The role of selenium in the various selenoenzymes and the mechanism of incorporation of selenocysteine in selenoprotein A and in formate dehydrogenase are the subjects of our ongoing studies.

PHS-6040
(Rev. 10-76)

Project Description

1. Anaerobic metabolism of certain amino acids with special references to the role of selenium, molybdenum, quinones, flavins and non-heme iron proteins in the electron transfer and phosphorylation reactions involved.

    a. Structure and function of the selenoprotein component of glycine reductase and its inter action with other protein components of the enzyme complex. Mode of biosynthesis of the selenium-containing moiety, a selenocysteine residue of the selenoprotein.

    b. Purification and characterization of additional components of the glycine reductase complex in order to determine the mechanism of the reaction and the coupled phosphorylation process.

2. Isolation and characterization of other seleno-enzymes and selenium containing t-RNAs.

    a. Formate dehydrogenases of Methanococcus vannielii and Clostridium sticklandii.

    b. Characterization and biochemical role of seleno-thiolase produced by Clostridium kluyveri and seleno-nicotinic acid hydroxylase produced by Clostridium barkeri.

    c. Mechanism of formation of tRNAs modified with selenium and their biochemical roles.

3. Methane biosynthesis from formate and acetate and the roles of vitamin B_{12} and 5-deazaflavin in the process.

Major Findings

(1) Continued studies on clostridial glycine reductase have dealt mainly with production of amounts of purified proteins (selenoprotein A, protein B and fraction C protein) sufficient for detailed mechanism studies. Additional evidence that protein B is a pyruvyl protein results from the work of Dr. Sue Neece (part time employee). Time course studies on the synthesis of selenoprotein A in intact cells indicate that this is a much slower process than seleno-tRNA formation (see later); no evidence for a stable precursor protein that is subsequently modified with selenium has been obtained. Once the biologically active selenoprotein is formed it is stable and remains in the cells even during prolonged incubation in the presence of antibiotics such as chloramphenicol, rifampicin, etc. that prevent cell multiplication.

(2) New bacterial selenoproteins (1) nicotinic acid hydroxylase of C. barkeri and (2) thiolase of C. kluyveri have been investigated in collaboration with G. Dilworth and M. Hartmanis, respectively (see their

individual reports). Nicotinic acid hydroxylase provides another example of a seleno enzyme that catalyzes an oxidation-reduction reaction whereas thiolase catalyzes carbon-bond condensation reactions and is the first example of a non-redox selenoprotein. The stability of selenium in the seleno-thiolase together with the ease of purification of the biologically active enzyme makes this protein a good candidate for studies of the mechanism of biosynthesis of selenoproteins. C. kluyveri grows in a simple, completely defined medium and thus studies with labeled potential precursors of the selenium containing moiety of the protein are reasonable to try.

Amino acid transfer nucleic acids specifically modified with selenium (seleno-tRNAs) which were first found in Clostridium sticklandii have been detected in other microorganisms, both anaerobic and aerobic. C. sticklandii, which is the richest source found so far, produces three readily-separable seleno-tRNAs. These materials, labeled with ^{75}Se, have been used to develop methods suitable for the isolation and identification of the seleno nucleoside(s) present in the modified tRNAs. Mild alkali digestion converted the ^{75}Se-tRNAs to a population of ^{75}Se-nucleotides that accounted for about 90% of the ^{75}Se. The yield of small fragments suitable for base analysis was 40% and partially degraded material could be further cleaved by additional treatment with alkali. Satisfactory recovery of ^{75}Se-labeled nucleotides after separation by thin layer chromatography indicates the suitability of the procedures for final isolation and identification. The chemical properties of the ^{75}Se moiety indicate attachment of Se to the ring of a purine or pyrymidine base.

Proposed Course of Research

(1) Studies on the structure of selenoprotein A of glycine reductase with especial regard to the location of the selenocysteine residue and the 2 cysteine residues in the polypeptide chain will be continued. Using antibodies to the protein and ^{32}P-labeled orthophosphate a search for phosphorylated protein A will be made.

The subunit structure and number of pyruvyl groups on protein B will be investigated. Fraction C protein will be purified from cells labeled with radioactive iron to see if this is indeed an iron protein.

(2) Studies on the identity or identities of Se-moieties of nicotinic acid hydroxylase and thiolase will be carried out. If thiolase contains selenocysteine, the biosynthesis of this protein will be studied in order to answer the question of mode of incorporation of this amino acid in proteins. Studies are underway to see if C. sticklandii or E. coli contains a tRNA specific for selenocysteine. Identification of the seleno-base(s) of seleno-tRNA's and the enzymic mechanism of their synthesis will be attempted.

(3) In collaboration with S. Yamazaki and L. Tsai the importance of the 5-deazaflavin dependent formate-NADP$^+$ oxidoreductase system of M. vannielii for methane biosynthesis will be examined. Attempts will be

made to see if the reduced 5-deazaflavin is used directly as reductant for the terminal reaction step in which a methyl group is reduced to methane. An alternative possibility that NADPH is a necessary intermediate will be tested. Current interest in biological formation of hydrocarbons makes these studies at the enzyme level of timely significance.

Honors

Secretary and Program Chairman of the American Society of Biological Chemists (end of 2nd to beginning of 3rd year of 3 year term).

Member of U. S. National Committee to IUB from the Biological Chemistry Division of the American Chemical Society (five year term).

Introductory Lecture at the German Branch of American Society of Microbiology, Saarbrucken, Germany, March 1980.

Plenary Lecturer at Second International Symposium on Selenium in Biology and Medicine, May 1980, Lubbock, Texas.

Public Health Service Superior Service Award, May 23, 1980.

Member of the Department of Energy Workshop to plan DOE sponsored research on genetics of anaerobic bacteria (April 1980).

Publications

J. B. Jones, Gregory L. Dilworth and T. C. Stadtman. Occurrence of selenocysteine in the selenium-dependent formate dehydrogenase of Methanococcus vannielii. Archiv. Biochem. Biophys. 195, 255-260 (1979).

J. B. Jones and T. C. Stadtman. Reconstitution of a formate-NADP$^+$ oxidoreductase from formate dehydrogenase and a 5-deazaflavin-linked NADP$^+$ reductase isolated from Methanococcus vannielii. J. Biol. Chem. 255, 1049-1053 (1980).

C.-S. Chen and T. C. Stadtman. Selenium-containing tRNAs from Clostridium sticklandii: Co-chromatography of one species with L-prolyl tRNA. PNAS 77, 1403-1407 (1980).

T. C. Stadtman. Selenium-Dependent Enzymes. Ann. Rev. Biochem. 49, 93-110 (1980).

J. J. Baker and T. C. Stadtman. Amino Mutases. Chapter for two volume treatise on "Vitamin B_{12}". Wiley & Sons. (in press).

T. C. Stadtman. Biological Functions of Selenium ....Invited review for TIBS (in press).

T. C. Stadtman, G. L. Dilworth and C.-S. Chen. Selenium dependent Bacterial Enzymes. Proc. of 3rd Int. Se and Te Meeting, Metz, France (in press).

T. C. Stadtman. Bacterial Selenoenzymes and Seleno-tRNAs. Proc. of 2nd Int. Symp. on Selenium in Biology and Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00206-21 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Stereochemical Studies of Enzymatic Reactions (5-Deazaflavin)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Lin Tsai	Research Chemist	LB	NHLBI
Other:	S. Yamazaki	Staff Fellow	LB	NHLBI

COOPERATING UNITS (if any)

C. Walsh, MIT, Cambridge, Massachusetts (Stereochemical Studies)

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.0	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A number of simple analogues of the 8-hydroxy-5-deazaflavin cofactor were prepared for enzymatic study with 8-hydroxy-5-deazaflavin-dependent NADP$^+$ reductase from Methanococcus vannielii. The stereochemical course of the reductase catalyzed reaction was established as S-stereospecific with respect to the pyridine nucleotide.

PHS-6040
(Rev. 10-76)

Project Objectives

8-Hydroxy-5-deazaflavin-dependent NADP$^+$ reductase, a homogeneous enzyme (Yamazaki) from the extracts of Methanococcus vannielii, catalyzes the reaction:

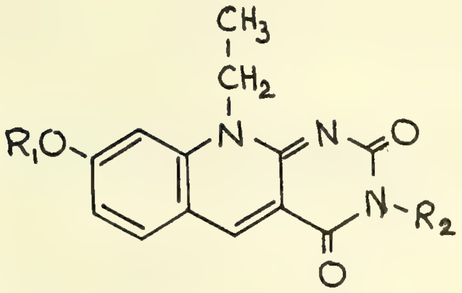
$$\begin{array}{l}\text{Dihydro-8-hydroxy-}\\\text{5-deazaflavin anion}\end{array} + \text{NADP}^+ \rightleftharpoons \begin{array}{l}\text{8-Hydroxy-}\\\text{5-deazaflavin}\end{array} + \text{NADPH} \qquad (1)$$

A simple analogue (Ia) of the aromatic chromophore of the 8-hydroxy-5-deazaflavin cofactor has been found to undergo the enzymic reaction, though at a slower rate than that of the natural cofactor. Thus, it is desirable to prepare other similar analogues in order to probe the structural requirements for enzymatic activity with respect to the 8-hydroxy-5-deazaflavin chromophore.

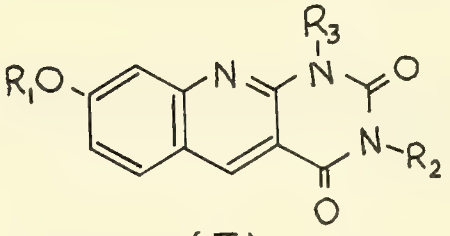
This reaction (equation 1) is similar to many other flavin-pyridine nucleotide redox systems, except that this is the first example of such a reaction involving a natural 5-deazaflavin instead of a flavin. It is, therefore, of interest to establish the mechanism of the hydride transfer process and the stereochemical course of the reaction.

Major Findings

The analogues of interest for the enzymatic studies are the methyl derivatives of structures (I) and (II).



(I)

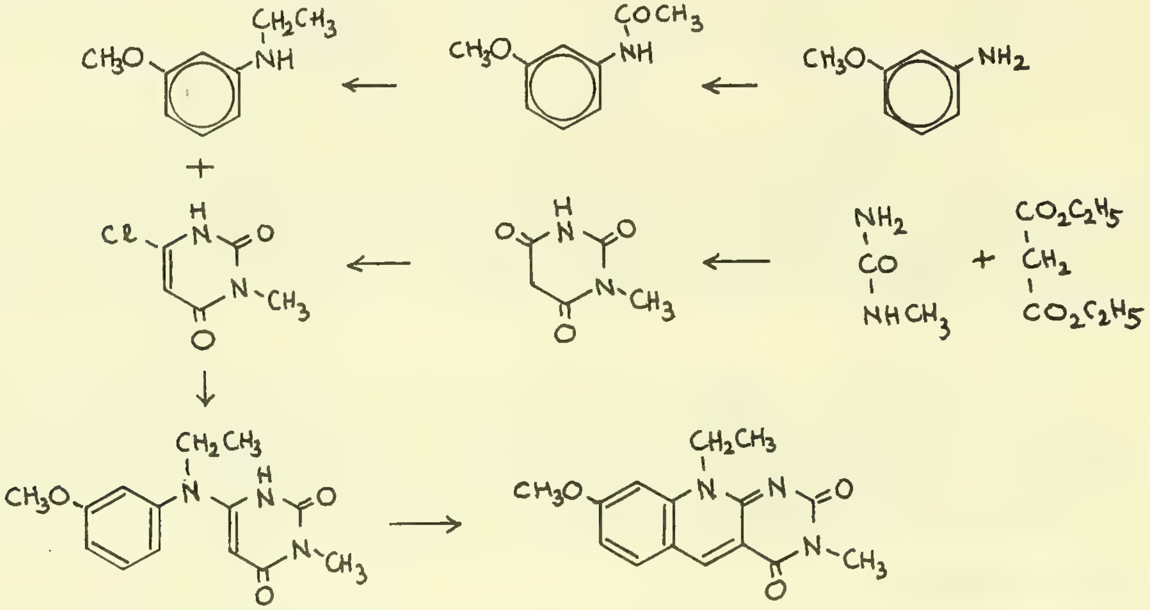


(II)

	R$_1$	R$_2$
(Ia)	H	H
(Ib)	H	CH$_3$
(Ic)	CH$_3$	H
(Id)	CH$_3$	CH$_3$

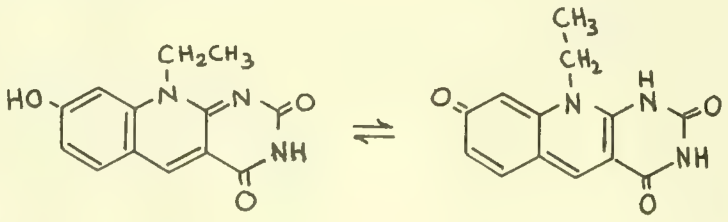
	R$_1$	R$_2$	R$_3$
(IIa)	H	H	H
(IIb)	H	CH$_3$	H
(IIc)	CH$_3$	H	H
(IId)	CH$_3$	CH$_3$	H
(IIe)	H	CH$_3$	CH$_3$

Attempted direct methylation of compounds (Ia) and (IIa) led invariably to mixtures of isomeric methyl and dimethyl derivatives which were very difficult to separate. In order to insure the integrity of each final product, specific synthesis was designed for each compound by first preparing the appropriately methylated starting components. For example, compound (Id) was synthesized as follows:



Through various combination of the two starting components, compounds (I b-d) and (II b-e) were thus prepared. Although the two steps, the condensation and the cyclization, were fundamentally the same in each preparation, it was somewhat surprising to note that the success of each preparation depended much on the choice of the reaction conditions for these two steps. The structural assignments of all these compounds were consistent with their spectroscopic properties.

Examination of the electronic spectra of these compounds at various pH's reveals their ionic and probably tautomeric states. For instance, the unsubstituted compound, (Ia), can exist in either the phenolic form (Ia) or the paraquinoid form (Ia)':

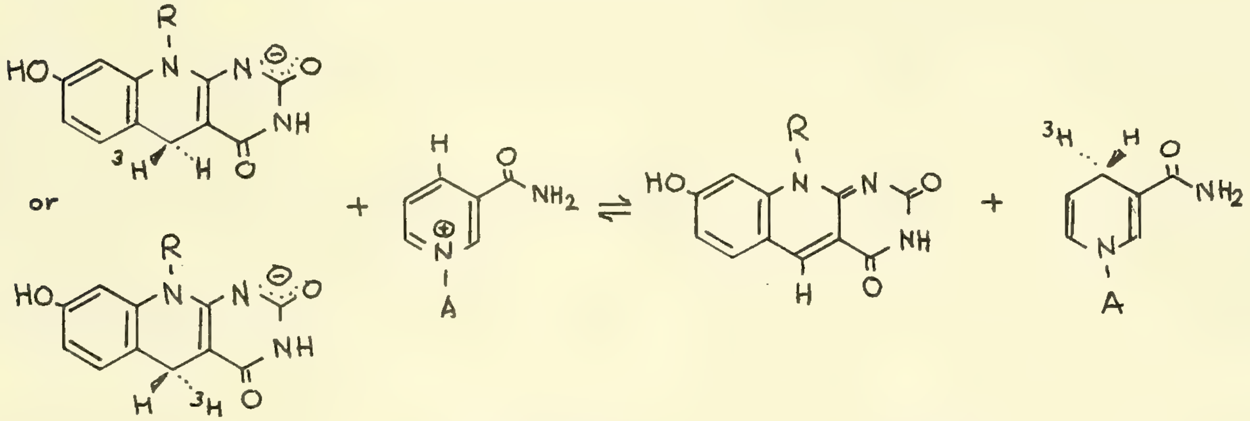


(Ia) (Ia)'

In compounds (Ic) and (Id), the 8-hydroxy function is replaced by the methoxy group, thus prohibiting the existence of the paraquinoid form. The similarity of the uv spectra of the four compounds (I a-d) at pH 4-6 suggests that the neutral state of (Ia) exists mainly in the phenolic form. This also demonstrates the lack of involvement of N_3-position in the tautomerism. Of the four compounds, only (Ia) and (Ib), whose 8-hydroxy function is not blocked by a methyl, show a red shift of the long wavelength band at pH > 9. This indicates that at high pH, the spectrum is that of an anion derived from the 8-hydroxy function. This interpretation is in agreement with that of Ghisla and Mayhew on 8-hydroxy-riboflavin. Similar consideration can be applied to the (II) series of compounds.

Comparison of compounds (I a-d) as substrate for the reaction (equation 1) catalyzed by 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase (Yamazaki) shows the absolute requirement of the free 8-hydroxy function for enzymatic activity and the indifference of the substitution at the N_3-position.

The stereochemical course of the reversible hydride transfer (equation 1) between the 8-hydroxy-5-deazaflavin cofactor and the nicotinamide coenzyme was studied (Yamazaki and Walsh) by using stereospecifically tritium-labeled substrates. The results of these experiments established a S-stereospecificity with respect to the nicotinamide coenzyme and the overall reaction can be represented as follows:



Proposed Course of Action

(1) The chemical and biochemical properties of the analogues will be studied with an aim to design an adequate derivative usable for affinity column chromatography.

(2) The determination of the absolute configuration of a chirally labeled 1,5-dihydro-8-hydroxy-5-deazaflavin will be attempted.

Publications

Yamazaki, S. and Tsai, L.: Properties of 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii. Abs. 490 71st American Society of Biological Chemists Meeting, June 1-5, 1980.

Yamazaki, S. and Tsai, L.: Purification and properties of 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii. (1980) J. Biol. Chem. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00211-07 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Cellular Regulation - Multiplemolecular Forms of Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
Others:	R. J. Hohman	Chemist	LB NHLBI
	P. Smyrniotis	Research Chemist	LB NHLBI
	S. G. Rhee	Research Chemist	LB NHLBI
	M. Wittenberger	Biological Laboratory Technician	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Native glutamine synthetase preparations containing an intermediate number (2 to 10) of adenylylated subunits are complex mixtures of molecules that differ from one another with respect to the number of adenylylated subunits they contain. By means of affinity chromatography on Affi-blue sepharose columns or on anti-AMP antibody columns, such preparations have been resolved into a series of more or less homogenous fractions, each containing a specified number of adenylylated subunits. In addition, multiple enzyme species containing the same number of adenylylated subunits, but which differ from one another in their affinities for ADP and/or sepharose bound Cibacron dye, have also been obtained. These presumably represent isomeric forms that differ in the distribution of adenylylated and unadenylylated subunits within the dodecameric structure.

Project Description

Objectives: From theoretical considerations, it can be demonstrated that glutamine synthetase in Escherichia coli can exist in up to 384 different forms that differ from one another by the number (0 to 12) and distribution of adenylylated subunits within the dodecameric structure (M.S. Raff and W.C. Blackwelder). Although experimental evidence has been obtained for the existence of multiplemolecular forms, previous efforts to separate these from one another by electrophoresis, ion exchange chromatography, or electrofocusing techniques have failed. Two new approaches to this problem were suggested by the following findings: (1) The affinity of ADP for unadenylylated subunits is about 1,000 times greater than its affinity for adenylylated subunits; (2) Cibacron Blue dye will bind to the ADP site on the enzyme and can be displaced by ADP; (3) The capacity of anti-AMP specific antibodies to precipitate adenylylated glutamine synthetase is a function of the average number and possibly also of the distribution of adenylylated subunits. The present study was undertaken to determine if the different glutamine synthetase species present in partially adenylylated glutamine synthetase preparations could be separated by means of affinity chromatography on sepharose columns to which either Cibacron Blue (Affi-blue gel columns) or anti-AMP antibodies were covalently attached.

Major Findings

(1) Separation of Multiplemolecular Forms of Glutamine Synthetase by Chromatography on Affi-blue Sepharose Columns. Native glutamine synthetase preparations, each at a different state of adenylylation, were adsorbed on Affi-blue speharose columns and were subsequently subjected to gradient elutions with buffer in which the concentration of ADP was varied exponentially from 0 to 1.5 mM. As the concentration of ADP was increased, there was a progressive increase in the state of adenylylation, $\bar{n}$, of the glutamine synthetase which was eluted from the column. A comparison of the ADP vs $\bar{n}$ profiles of the different glutamine synthetase preparations subjected to affinity chromatography under identical conditions showed that the concentration of ADP required to elute glutamine synthetase with a given number of adenylylated subunits is an inverse function of the initial state of adenylylation of the native enzyme mixture used.

The data show that native enzyme preparations are mixtures of enzyme species that differ from one another by the number of adenylylated subunits they contain, and that these different species can be separated from one another by affinity chromatography on Affi-blue columns. Additionally, they show that all enzyme species containing identical numbers of adenylylated subunits (isomeric forms) are not the same, and that the concentrations of a given isomeric form varies with the initial state of adenylylation of the enzyme. It is presumed that these isomeric forms differ from one another by the distribution of adenylylated subunits in the dodecameric molecule and that this distribution may be a function of the relative activities of the enzyme cascades that lead to adenylylation of glutamine synthetase on the one hand, and to its deadenylylation on the other.

(2) Separation of Variously Adenylylated Glutamine Synthetase Species by Affinity Chromatography on Immobilized Anti-AMP Antibody Columns. Previous studies (see last year's Annual Report) showed that the immunoprecipitability of glutamine synthetase by AMP-specific antibodies varies directly with the average state of adenylylation over the range of $\bar{n} = 4$ to $\bar{n} = 10$. Based on this characteristic, a procedure was developed for the partial separation of variously adenylylated glutamine synthetase species by affinity chromatography on sepharose columns of immobilized anti-AMP antibodies. Glutamine synthetase which on the average contained six adenylylated subunits per molecule was adsorbed on the antibody column and then eluted with buffer containing a gradient of AMP. The state of adenylylation of the glutamine synthetase species eluted from the column increased from 2 to 10 as the concentration of AMP in the elution buffer was increased from 0 to 20 mM.

These results attest to the heterogeniety of native glutamine synthetase preparations.

The existence of isomeric forms of glutamine synthetase (i.e., forms with the same number but different distributions of adenylylated subunits) is suggested also by the results of immunotitration experiments. These studies show that anti-AMP antibodies react with some species of adenylylated glutamine synthetase in a given enzyme preparation to form soluble immune complexes, whereas they react with others to form precipitable complexes. It is proposed that the distribution of adenylylated subunits in some glutamine synthetase molecules favors intramolecular reactions with the antibodies to preclude lattice formation, whereas the distribution of adenylylated subunits in other species of enzymes favor intermolecular cross-linking, and therefore the generation of precipitable complexes.

Proposed Course of Action

The above studies will be continued. By means of Affi-gel column chromatography, efforts will be made to isolate large quantities of two different isomeric forms of glutamine synthetase which have the same number of adenylylated subunits. The immunoprecipitability of these isomers by anti-AMP antibodies will be compared, and detailed studies of their catalytic activities and their susceptibilities to feedback inhibition by various end products of glutamine metabolism will be examined.

Publications

Stadtman, E.R., Hohman, R.J., Davis, J.N., Wittenberger, M., Chock, P.B., and Rhee, S.G.: Subunit Interactions of Adenylylated Glutamine Synthetase. In The Lipmann Symp. on Concepts of Chemical Recognition in Biology, Grignon, France, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00212-09 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Ammonia-Assimilatory Enzymes in Escherichia coli K12

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mary Anne Berberich	Research Chemist	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.0	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Genetic and biochemical studies with enterobacteria suggest that the regulation of nitrogen metabolism in these organisms is quite complex. The NH_3-assimilatory enzymes, as well as some amino acid transport systems and catabolic enzymes, appear to be involved in the process. Because of its unique biochemical properties and its important role in nitrogen metabolism, glutamine synthetase is of primary interest. The purpose of this work is to study, via genetic and biochemical techniques, the mechanism by which the synthesis of glutamine synthetase is controlled in Escherichia coli K12. Special attention is focused on identifying the elements of this control and on determining whether they are specific for the regulation of glutamine synthetase or whether they might represent effectors in the regulation of the class of NH_3-assimilatory enzymes within the general scheme for nitrogen control.

Project Description

Objectives: 1. Selection of, and genetic studies on, mutants of *Escherichia coli* K12 manifesting alterations in the amount or regulation of the NH_3-assimilatory enzymes: glutamine synthetase (GS), glutamate synthase (GAT), and glutamate dehydrogenase (GDH).

2. Biochemical characterization of these genetic effects.

3. Physiological studies with isogenic wild type and mutant strains where the levels of these enzymes are measured under a variety of growth conditions in order to ascertain (a) whether a regulatory interrelationship exists for these three enzymes, and (b) whether one or all of them play a regulatory role within the larger framework of nitrogen control.

Major Findings

I. Genetic Studies

(a) *Selection of mutants*. It was reported earlier that mutants capable of utilizing L-glutamate as carbon source could be isolated from *E. coli* K12 strains N99, 3000, and Lederberg at frequencies ~ 10^{-7} and that this glutamate utilizing trait was 100% associated with a pl resistance trait in reversion studies. At this point, the loss of the gal$^-$ marker carried by N99 was noted and temporarily put aside, since a fundamental change involving the cell surface, which might also effect the gal kinase, was postulated to explain the behavior of these isolates. However, when subsequent attempts to isolate mutants of this type from other suitably marked strains proved unsuccessful, it became necessary to reexamine the original glutamate carbon-utilizing isolates according to other biochemical criteria. On the basis of oxidase activity and lack of glutamate decarboxylase activity, these lac$^+$, peritrichously flagellated isolates were identified as members of the closely related genus, Erwinia. This characterization was also consistent with the yellow pigmentation and 30°C growth temperature optimum previously reported. Their presence was traced to a contaminated batch of sterile water. Moreover, none of these showed derepressed levels of glutamine synthetase when grown on glutamate carbon with NH_4Cl as nitrogen source. The one glutamate carbon-utilizing isolate which did synthesize glutamine synthetase constitutively in presence of NH_4^+ nitrogen was subsequently shown to be a Pseudomonas. Like N99, this strain carried a high level streptomycin resistance marker and, like the "revertants" and the Erwinia described above, could be traced to the same batch of sterile water. Although the galactokinase marker of N99 has remained intact throughout many selective procedures, the involvement of various transport systems with nitrogen control which might indirectly affect this trait makes it desirable to install some other character which would enable easy and rapid identification of the strain. Accordingly, a selection for a histidinol dehydrogenase mutation (hisD$^-$) in N99 is in progress. No glnF$^-$ has, as yet, been obtained directly from N99 by ICR mutagenesis. Other methods are in progress.

(b) *Mapping experiments*. Both the Erwinia and Pseudomonas strains could be lysogenized by ϕ pl CAM clr 100, and would, on induction, produce reasonably high titres of lytic phage. In the case of the Pseudomonas, probably due to host modification of the virus, these were not transducing particles when used with E. coli recipients. Although the phage produced from the Erwinia would transduce E. coli his^{-} and arg^{-} to his^{+} and arg^{+}, respectively, lack of transfer of the glutamate carbon-utilizing trait suggests that more than one unlinked gene is involved.

(c) Construction of isogenic strains carrying glnA or glnD genes on plasmids. Mutants affecting UR-UTase (glnD) map at a site distinct from glnA (85') close to DAP-D (4'), have very low levels of highly adenylylated GS, and are dependent on exogenous glutamine for growth. The glnD locus appears to specify both UR and UTase activity (see publication listed below) and appears to play a regulatory role in the expression of glnA. Since the addition of D-glutamate to cells growing exponentially with NH_4Cl as nitrogen source evoked increases in the specific activities of GS, GAT, and GDH, it seemed reasonable to examine whether the ratio of glnD product to glnA (or glnG) product would have an influence on this effect. Since most of the physiologic studies in this laboratory have been done with strain W3102 (N99), it was necessary to construct a series of isogenic strains so that meaningful comparisons could be made in this type of experiment. For this purpose, strains of E. coli from the Clarke-Carbon collection were used as donors of the appropriate plasmids. Table 1 summarizes these derivatives.

Table 1

Strain	Relevant phenotype	Genotype	Parent	Source
N99	--	gal^{-} sm^{r}	wild type (K12-W3102)	M. Gottesman
N100	--	gal^{-} sm^{r} rec^{-}	N99	M. Gottesman
MB1	gln^{-}	glnD-21	N99	ICR mutagenesis
MB2	gln^{+} ColE1^{r}	glnD-21 ColE1 glnD^{+}	MB1, JA200/ pLC 38-39	Conjugation
MB3	gln^{+} ColE1^{r}	glnD-21 ColE1 glnA^{+}	MB1, JA200/ pLC 41-35	Conjugation
MB21	gln^{-}	glnA-67	N99	DES mutagenesis
MB22	gln^{+} ColE1^{r}	glnA-67 ColE1 glnA^{+}	MB11, JA200/ pLC 41-35	Conjugation
MB23	gln^{-*} ColE1^{r}	glnA-67 ColE1 glnD^{+}	MB21, JA200/ pLC 38-39	Conjugation
N991	ColE1^{r}	ColE1 glnA^{+}	N99, JA200/ pLC 41-35	Conjugation
N1001	ColE1^{r}	ColE1 glnA^{+}	N100, JA200/ plC 41-35	Conjugation
N1002	ColE1^{r}	ColE1 glnD^{+}	N100, JA200/ pLC 38-39	Conjugation

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[*] It should be noted that MB23 is not glutamine independent and is, rather, glutamine sensitive as compared to MB21 or N1002 and MB2. This property may allow for a positive selection, in MB21, of more than 1 episome.

II. Biochemical Characterization of Strains

(a) Standardized conditions. Enzymes were assayed in crude extracts prepared from cells growing under standardized conditions: early log phase (between 5 X 10^7 and 5 X 10^8 cells/ml) with growth and enzyme levels monitored over a period which included one to two mass doublings. In addition to the presence of excess ammonia, the level of GS could be further repressed by addition of glutamine to the medium. Therefore, "repressing" medium = glucose (11 mM) mineral salts, ammonia (20 mM), and glutamine (3 mM). "Derepressing" medium = glucose (11 mM) mineral salts, glutamine (10 mM). With N99, the average specific activity observed for glutamine synthetase during "repressing" growth is .198 and that during "derepressing" growth is 1.22.

(b) Downshift experiments. Cells growing exponentially in repressing medium were collected, washed, and resuspended in derepressing medium. (1) With N99, it could be shown that the initial rate of increase in GS level was constant over a 90 minute period during which the specific activity increased approximately 4-fold. During this period, the specific activities of GAT and GDH did not change, while the increase in $\bar{n}$ value paralleled the increase in GS activity. (2) The ^{32}P labeling pattern of nucleotides during this period may indicate a negative effect of ppGpp on the rate of this process. This observation is being pursued. (3) The level of GS activity is elevated in strain MB22, as compared to N991 and N1001, both in glycerol minimal salts medium and in derepressing medium following a 30 minute downshift as described above. In the case of MB22, $\bar{n}$ increased from 2.0 to 6.8 during this downshift period, whereas $\bar{n}$ for N991 increased only slightly. This observation will be pursued.

III. Physiological Studies

The failure to express the multiple potential of the glnA containing-plasmids could be interpreted as a limitation in the availability of some positive effector. On the other hand, lack of increase due to glnA copies could reflect the limit imposed by a closely regulated attenuation process involving the intracellular level of glutamine. It is highly likely that both processes are operating simultaneously and that the resultant level of GS reflects the state of balance between the positive and negative process. Because of the effect of glnD$^-$ mutation upon the expression of glnA, it appeared that the effects of D-glutamate addition, when examined in strains differing with respect to the ratio of glnD product to glnA product, might provide some insights into these processes. Enzyme levels in isogenic strains (see Table 1) growing in glycerol-minimal salts-ammonia were compared after a 60 minute exposure to D-glutamate. When D-glutamate is added to N99 growing exponentially in excess ammonia, the following are observed:

1. Increase in all three NH_4^+ assimilatory enzymes specific for D-glutamate, i.e., no effect of D-valine, D-alanine, D-aspartate.

2. D-glutamate addition elicits an increase in permeabilized and unpermeabilized cells. In permeabilized cells, L-glutamine at the same concentration, decreases effect by 50%, whereas L-glutamate completely eliminates the effect.

3. Rate of increase of GS and GAT identical (~ 2-fold in 90 minutes), whereas the faster rate of increase of GDH (~ 3-fold in 90 minutes) might indicate a hierarchy of response to the same stimulus.

4. No effect of D-glutamate addition during NH_4^+ limited growth.

A summary of the results with the isogenic plasmid-bearing strains follows below:

A. Glutamate dehydrogenase

1. Specific activity increases 73% within 60 minutes in strain N99.

2. Greater increases in specific activity are observed with strains carrying $glnA^+$ plasmids N1001 (152%); MB3 (106%); MB22 (117%).

3. All strains examined showed increased levels of GDH following D-glutamate addition, except for strain MB21 ($glnA^-$).

4. D-glutamate induction of GDH appears independent of glnD product and dependent on either: (a) activity of GS (MB21 is CRM^c) or, (b) presence of unaltered glnA (or glnG) product.

B. Glutamine synthetase

1. Total specific activity increases 19% within 60 minutes in strain N99.

2. Greater increases in specific activity are observed for wild type strains carrying $glnA^+$ plasmids: N991 (47%); N1001 (50%); and for a $glnD^-$ strain carrying a $glnD^+$ plasmid, MB2 (38%; where the initial specific activity is GS = 0.51).

3. The increase in GS elicited by D-glutamate appears independent of the state of adenylylation of the GS.

The above data suggest a reciprocity between the product of the glnD gene and a product of the glnA region. The growth of both $glnD^-$ and $glnA^-$ mutants is inhibited by L-glutamate. It has been noted that $glnD^-$ mutants give rise to L-glutamate resistant revertants which have a GS constitutive phenotype and map very close to glnA. In the absence of D-glutamate, elevated specific activity of GS is observed for $glnD^-$ and $glnA^-$ strains bearing $glnA^+$ plasmids (0.44 and 0.50, respectively).

The mechanism by which D-glutamate elicits these effects is obscure at present, but further studies planned with $glnF^+$ plasmid-bearing strains might help to clarify which elements of the GS regulatory system are involved.

Proposed Course of Action

1. Continue with selection for $glnF^-$ in strain N99.

2. Proceed with screening of Carbon collection to identify $glnF^+$-bearing plasmid by correction of gltB defect.

3. Construct ColE1 $glnF^+$ isogenic strains for study of D-glutamate effect relative to glnA and glnD.

4. Continue to explore hierarchy of nitrogen control for GS, GAT, and GDH.

5. Compare the D-glutamate, addition response and "derepressing" downshift systems.

6. Continue attempts to define genetic/physiologic conditions which will permit one to determine whether the NH_3-assimilatory enzymes are controlled, as a class, by induction, derepression, or by a complex attenuation scheme.

Publications

Garcia, E., Federici, M.M., Rhee, S.G., and Berberich, M.A.: Glutamine Synthetase Cascade: Enrichment of Uridylyltransferase in *E. coli* Carrying Hybrid ColEl Plasmids. Arch. Biochem. Biophys. 203, August 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00217-05 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of In vitro Assembly of Microtubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donita L. Garland	Staff Fellow	LB NHLBI
Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of the effects of cyclic nucleotides on the regulation of microtubule polymerization has been continued.

Adenosine, inosine, and guanosine stimulate the phosphorylation of two proteins with molecular weights of approximately 63,000 and 29,000 from 100,000 X g supernatants prepared from porcine brain. The reaction was studied in terms of stability of the phosphate bond, metal ion requirements, nucleoside and nucleotide specificity and mechanism. The phosphorylated proteins are presently being purified.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The continuing focus of this study has been on the role of cyclic nucleotides in the regulation of tubulin activity. We previously showed that cAMP in the presence of sodium fluoride inhibited the in vitro assembly of microtubules from crude supernatants. The present objectives are to purify and characterize the components involved in the cAMP-dependent inhibition of polymerization of tubulin.

In addition, we plan to further characterize the protein phosphorylation in brain supernatant that is stimulated by inosine and adenosine. The protein(s) will be purified and characterized.

Major Findings

(1) A new method for assaying polymerization of microtubules was developed. This method utilizes the colchicine binding properties of tubulin and has the advantages that it is rapid and directly quantitates the tubulin incorporated into microtubules.

The method has been modified so that the assay time is significantly reduced and the precision is increased.

(2) The charcoal method for assaying colchicine binding to tubulin has been modified. This allows much more accurate measurements of the initial phase of colchicine binding reaction. Using this method, we have demonstrated a biphasic binding curve. It is not known whether this reflects another species of tubulin or a more complex binding scheme, however, the former seems more likely.

(3) The nucleosides, adenosine, inosine, and guanosine, but not cytidine, stimulate the incorporation of the γ-phosphate of ATP into TCA precipitable protein from 100,000 X g supernatant prepared from porcine brain. The stimulation is 2 to 4 times that observed in the presence of cAMP. A similar stimulation is seen after the supernatant has been gel filtered on P10.

The phosphorylation reaction is time and temperature dependent; maximum labeling is seen at 5-10 minutes at 30°C. The kinetics of the reaction differ when adenosine and inosine are used. While the same maximum level of phosphorylation is reached, the response is sigmoidal in the presence of adenosine, and hyperbolic with inosine. AMP, IMP, and XMP increase the lag time.

Adenosine is rapidly converted to inosine (and AMP) in these preparations. The rate of conversion to inosine is compatible with the hypothesis that the observed lag is the time required to build up the concentration of inosine. In the presence of EHNA, an inhibitor of adenosine deaminase, there was no stimulation of phosphorylation. These two pieces of evidence suggest that inosine is the compound which stimulates the protein phosphorylation.

The labeling of the protein is not due to tightly bound ATP. ^{3}H-ATP was not incorporated. The studies on the stability of the phosphate label suggest the formation of an acyl phosphate bond. The label is very labile above pH 8.0, but stable at neutral pH and down to pH 4.0.

There is no stimulation of phosphate incorporation when Pi is substituted for ATP. Hypoxanthine does not stimulate phosphorylation. Inosine is not incorporated into the TCA precipitable protein. The reaction is inhibited by GTP.

The reaction was monitored in the presence of Mg^{++}, Mn^{++}, Ca^{++}, and EDTA. In the presence of Mg^{++}, Ca^{++}, and EDTA, there was a similar stimulation by inosine in terms of total pmoles of phosphate incorporated. The reaction rate is much slower in EDTA.

When the proteins are separated by SDS-polyacrylamide gel electrophoresis and autoradiography alone, there are three proteins that are labeled in the presence of inosine. These have molecular weights of approximately 63,000, 37,000, and 29,000. The relative proportion of phosphate incorporated into these appears to vary with the experimental conditions. With Mg^{++} present, the 29,000 Dalton protein is heavily labeled with a small increase in the labeling of the 63,000 Dalton protein. With Ca^{++} present, the 63,000 Dalton protein is most heavily labeled, with the 29,000 and 37,000 Dalton proteins also labeled. With EDTA present, only the 63,000 and 37,000 Dalton proteins are labeled. The inosine and Mg^{++} concentrations also seem to affect the pattern of labeling.

Proposed Course of Action

The following studies will be extended:

(1) Identify and purify the proteins that are phosphorylated or dephosphorylated in the presence of cAMP and fluoride in the crude supernatants and determine how they change the activity of tubulin.

(2) Purify the protein that is labeled with the γ-phosphate of ATP in the presence of inosine. Attempt to identify the protein.

Relevance to Medicine

The study of the function and cellular regulation of microtubules has widespread benefits for both medicine and the understanding of cellular function. Microtubules have been implicated in numerous cell processes and functions, including the control of hormonal activation of adenylate cyclase. The exact function of microtubules in each of these is still not clear. Since microtubules are required for mitosis, they are a good point for control of malignant cell growth. Likewise, microtubules are important in wound healing and in control of lyosome function. The latter is particularly important in pathogenesis of gout, arthritis, and periodontal disease. Other cases where a defect in control or function of microtubules contributes to diseases are: Alzheimer's disease, lung dysfunction, infertility in cases of nonmotile cilia and flagella, and Chediak-Higashi syndrome.

The importance of studying the effects of adenosine is illustrated by its implication in numerous cellular phenomena. Adenosine is purported to be a neurotransmitter, and it has a role in controlling the synthesis of cAMP. Aberrations in adenosine metabolism have resulted in diseases such as immunodeficiency disease and herediatry hemolytic anemia.

In contrast, inosine has no known similar effects on the cell. While inosine decreases cell damage in kidney, myocardium, and red blood cells, this effect is most likely due to the subsequent increase in adenine nucleotides. Thus, it is of interest to determine which enzyme inosine is increasing the phosphorylation of and determine if this is a unique activity of brain.

Publications

Garland, D.L.: cAMP Inhibits the In vitro Assembly of Microtubules. Arch. Biochem. Biophys. 198: 335-337, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00224-03 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enzyme Mechanism and Regulation (title modification)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Charles Y. Huang	Research Chemist	LB NHLBI
	Jerry H. Wang	Expert Consultant	LB NHLBI
Others:	Vincent Chau	Staff Fellow	LB NHLBI
	P. Boon Chock	Research Chemist	LB NHLBI

COOPERATING UNITS (if any)
H.N. Jayaram, Laboratory of Toxicology, National Cancer Institute, NIH

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.3	3.1	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The activation constant of cyclic nucleotide phosphodiesterase by calmodulin has been determined by several methods. No cooperativity was detected in the interaction of these two proteins. Two calmodulin binding sites per phosphodiesterase molecule are demonstrated by the Job plot.
(2) Ca^{2+} binding to calmodulin has been carried out by following the intrinsic Tyr fluorescence of calmodulin or by using the fluorescence change of dansylated troponin C as an indicator for the free Ca^{2+}. Initial positive cooperativity followed by negative cooperativity in Ca^{2+} binding was revealed in these studies.
(3) Kinetic studies showed that all four Ca^{2+} sites are required for forming the activated phosphodiesterase-calmodulin complex. A 100,000-fold increase in the affinity between phosphodiesterase and calmodulin can be achieved by a 20-fold preferential binding of each of the four Ca^{2+} to the protein-protein complex. The strong cooperativity of the four Ca^{2+} sites also permits the inactivation of phosphodiesterase to be turned on and off by a small change in Ca^{2+} concentration.

Project Description

Objectives:

(1) To gain knowledge of the regulatory and catalytic mechanism of enzymes, specifically those regulated by calmodulin.

(2) To develop or improve methods and theories applicable to the study of enzyme mechanisms.

Major Findings

(1) The activation constant, K_a', of cyclic nucleotide phosphodiesterase (PDE) by the fully liganded calmodulin (CM) and the stoichiometry of interaction between PDE and CM were determined so that a model could be constructed to test the mechanism of Ca^{2+} activation of PDE by CM.

K_a' was determined from the profile of activated PDE activity (observed initial rate minus the basal activity, i.e., PDE activity in the absence of Ca^{2+}) as a function of total CM concentrations according to the following equation (cf. Huang's Annual Report, 1977-78):

$$CM_{0.5} = K_a' + 0.5\ (PDE)_o \qquad (1)$$

where $CM_{0.5}$ is the total CM concentration at 50% activation; $(PDE)_o$ is the total PDE concentration, and $K_a' = K_a\ (K_{m6}/K_{m1})$. K_a is the dissociation constant for the $PDE \cdot CM \cdot Ca_4^{2+}$ complex; K_{m6} is the Michaelis constant for cyclic AMP (cAMP) for the $PDE \cdot CM \cdot Ca_4^{2+}$ complex; and K_{m1} is the Michaelis constant for cAMP for PDE. The K_a', K_{m6}, and K_{m1} obtained at pH 7.0, 25°C are 1.14×10^{-10} M, 3.0×10^{-5} M, and 1.5×10^{-4} M, respectively. From these data, a value of 5.7×10^{-10} M for K_a is obtained. No cooperativity was discernible from the activation curve.

The extent of activation and the affinity of PDE for CM vary with pH and temperature. At pH 8, 30°C, a higher activation factor (6-8-fold over the basal activity compared with 3-4-fold activation at pH 7, 25°C) and a higher K_a' (1.03×10^{-9} M) were observed. The validity of Equation 1 was confirmed when the values of $CM_{0.5}$ obtained at several PDE concentrations were plotted against total PDE concentrations. The slope of such a plot, 0.46, is very close to the theoretical value of 0.5.

To further verify the magnitude of K_a', the on-off rate constants for the $PDE \cdot CM \cdot Ca_4^{2+}$ complex formation were measured. The off-rate constant, evaluated by the addition of excess CM binding protein to pull CM away from the complex, has been reported to be about $3 \times 10^{-3}\ S^{-1}$ at pH 7, 30°C (Huang's annual report, 1977-78). The on-rate constant was determined by mixing equimolar PDE and $CM \cdot Ca_4^{2+}$, and following the rate of activation at a saturating level of cAMP. The protein concentrations were at least five times higher than K_a' such that the bimolecular reaction could be treated as an irreversible process. This process can be described by the following equations:

$$v = V_f - (V_f - V_b)/(1 + k_1 E_o t) \qquad (2a)$$

and $$P = V_f t - [(V_f - V_b) \ln (1 + k_1 E_o t)]/k_1 E_o \qquad (2b)$$

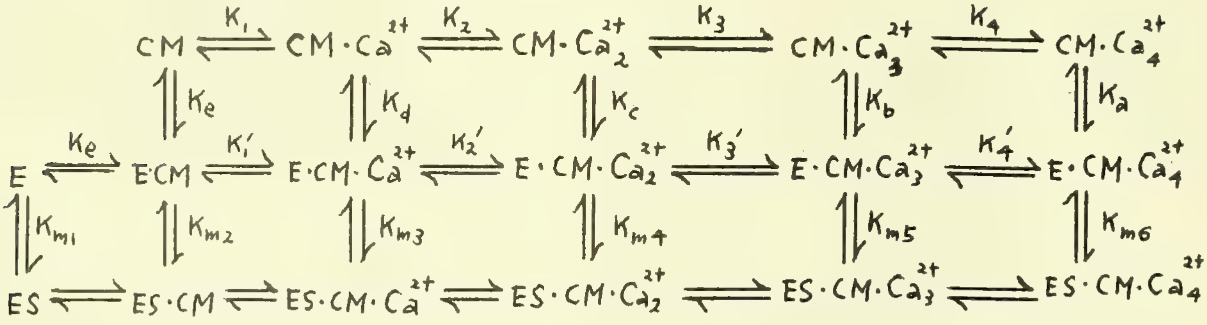
where V_f and V_b are the final and basal velocities; k_1 is the bimolecular rate constant; and P is the product. The second order rate constants obtained from three separate experiments using different protein concentrations agreed with one another quite well. The resultant value, $4 \times 10^6\ M^{-1}\ s^{-1}$, permits the calculation of a K_a' of 0.8×10^{-9} M from the ratio of off-rate and on-rate constants, which agrees quite well with the K_a' of 1.03×10^{-9} M determined from activation curves. At pH 7.0, 25°C, the on-rate constant similarly determined also is about $4 \times 10^6\ M^{-1}\ s^{-1}$, indicating that the tighter binding affinity for the PDE and $CM \cdot Ca_4^{2+}$ is due mainly to a slower off-rate constant.

Knowledge of K_a' allows us to determine the stoichiometry of interaction between PDE and CM by the continuous variation approach (Job plot). The number of CM bound per PDE dimer, n, is computed according to the following expression:

$$X/Y = (K_a' + n\ C_o)/(K_a' + C_o)$$
$$= n \text{ if } C_o \text{ is large} \qquad (3)$$

where X and Y are the mole fractions of CM and PDE at the intersection point of the two limiting slopes in the activation vs mole fraction plot, and C_o is the sum of PDE and CM concentrations maintained at a constant value. The C_o used in the experiments were greater than 10 K_a' to insure the validity of the value of n determined by this method. When the molar concentration of PDE was expressed in terms of subunits, an n number of 1.13 was obtained; whereas when PDE concentration was expressed in terms of dimers, n = 2.0. These values showed that there is one CM binding site per PDE subunit.

From the above experiments, it is concluded that there are two equal, noninteracting CM sites on the dimeric PDE. Thus, the PDE system can be treated as a monomeric enzyme and the following general scheme for the activation of PDE can be written:



Scheme I

In this scheme, K_1, K_2, K_3, K_4 = dissociation constants for the $CM \cdot Ca^{2+}$ complexes; K_1', K_2', K_3', K_4' = dissociation constants for the $PDE \cdot CM \cdot Ca^{2+}$ complexes; K_a, K_b, K_c, K_d, K_e = dissociation constants for the PDE and liganded CM complexes; K_{m1}, K_{m2}, K_{m3}, K_{m4}, K_{m5}, K_{m6} = Michaelis constants for cAMP for the various PDE species. It should be noted that the scheme merely describes the degree of saturation of Ca^{2+} for CM, E·CM, and ES·CM complexes. Sequential binding of Ca^{2+} is not implied.

(2) To study the Ca^{2+}-dependent activation of PDE by CM, the dissociation constants for the $CM \cdot Ca^{2+}$ complexes must be determined. Although several laboratories have reported on the binding of four moles of Ca^{2+} per mole of CM, the mode of Ca^{2+} binding has remained a subject of controversy. Thus, Ca^{2+} binding to CM was reinvestigated under the experimental conditions of our mechanistic studies: pH 7.0, 25°C, in the presence of 5 mM Mg^{2+}, and with free Ca^{2+} buffered by 1 mM EGTA. It has been reported that the Tyr fluorescence of CM was enhanced upon binding of Ca^{2+}. This property was utilized as an indicator of Ca^{2+} binding in our studies. However, the fluorescence change due to the successive binding of Ca^{2+} may not increase linearly. To circumvent this problem, a standard curve of Tyr fluorescence as a function of bound Ca^{2+} was constructed by the following approach: a high concentration of CM sample, 4×10^{-4} M, which is 10-100 times higher than the reported Ca^{2+} dissociation constants, was used such that the Ca^{2+} added to the sample up to four equivalent amounts was completely bound (EGTA buffer was not used). Assuming no interference by the presence of EGTA, one can determine the amount of Ca^{2+} bound by comparison of the percent fluorescence change observed at various free Ca^{2+} concentrations (maintained by the Ca^{2+}-EGTA buffer) with that of the standard curve. In addition, in a separate experiment, the large fluorescence change due to Ca^{2+} binding to dansylated troponin C reported by Potter was utilized as an indicator for the free Ca^{2+} concentration (EGTA buffer not used). The dansylated troponin C was present in small amounts such that the equilibrium between Ca^{2+} and CM was not disturbed. The Ca^{2+} titration curves obtained by the two methods described above were found to coincide with each other, provided a stability constant of $10^{10.4}$ for the $Ca^{2+} \cdot EGTA$ complex reported by Ogawa, instead of the 10^{11} reported by Schwarzenbach for unbuffered (pH) solutions, was used to compute free Ca^{2+} concentrations in the EGTA-buffered experiments. This observation supports Ogawa's finding that the formation constant of Ca^{2+}-EGTA is somewhat lower in a buffered solution.

Scatchard plot of our Ca^{2+} binding data showed four Ca^{2+} sites per CM molecule and displayed unmistakable positive cooperativity. Computer fitting of the data yielded the following dissociation constants: $K_1 = 7.5 \times 10^{-5}$ M, $K_2 = 2.7 \times 10^{-6}$ M, and $K_3 = K_4 = 3.0 \times 10^{-5}$ M. The initial positive cooperativity and subsequent negative cooperativity revealed by these constants are in agreement with the observations previously reported by Crouch and Klee. The dissociation constants also are similar in magnitudes to those obtained in the presence of 3 mM Mg^{2+} by Crouch and Klee.

(3) Kinetic studies on the activation of PDE by CM as a function of Ca^{2+} concentration have been reported by several laboratories, but detailed, systematic analysis is still lacking. The highly cooperative Ca^{2+} activation curve has prompted some workers to propose that 3-4 Ca^{2+} must be bound for CM to exert its activating effect on PDE. However, these workers based their proposal on the amount of Ca^{2+} bound to the $PDE \cdot CM$ complex, which was calculated by using Ca^{2+} dissociation constants obtained from Ca^{2+} binding to CM alone. Since the interaction between PDE and CM in the absence of Ca^{2+} is very weak (i.e., K_e is rather large, cf. Scheme I) and K_a is on the order of 10^{-9} M, it is obvious that Ca^{2+} binding to CM in the presence of PDE must be considerably tighter (otherwise K_a will equal K_e). Clearly, the inappropri-

ate calculation of the amount of bound Ca^{2+} does not permit these workers to make such a conclusion. Any quantitative analysis of the mechanism of PDE activation must be approached from kinetic considerations.

From Scheme I, a rate equation for the activation of PDE by CM and Ca^{2+} can be derived. To avoid excessive mathematical treatment, we shall go directly to the case in which the $PDE \cdot CM \cdot Ca_4^{2+}$ is the only activated species and $CM_o >> PDE$:

$$\Delta v = \frac{\Delta V_m\ (CM_o)\ (K_{m1}/K_e\ K_{m6})\ (\Phi_1/\Phi_2)}{1 + (CM_o/K_e)\ (\Phi_3/\Phi_2)} \tag{4}$$

where $\Delta v = v$ - basal activity

$\Phi_1 = C^4/K_1'\ K_2'\ K_3'\ K_4'$ $\quad$ ($C = Ca^{2+}$ concentration)

$$\Phi_2 = 1 + C/K_1 + C^2/K_1\ K_2 + C^3/K_1\ K_2\ K_3 + C^4/K_1\ K_2\ K_3\ K_4$$

$$\Phi_3 = K_{m1}/K_{m2} + (K_{m1}/K_{m3})\ C/K_1' + (K_{m1}/K_{m4})\ C^2/K_1'\ K_2'$$
$$+ (K_{m1}/K_{m5})\ C^3/K_1'\ K_2'\ K_3' + (K_{m1}/K_{m6})\ C^4/K_1'\ K_2'\ K_3'\ K_4'$$

Double reciprocal plot of $1/\Delta v$ vs $1/CM_o$ will yield the following parameters:

$$\text{slope} = (K_e\ K_{m6}/K_{m1})\ (\Phi_2/\Phi_1) \tag{5a}$$

$$\text{and y intercept} = (K_{m6}/K_{m1})\ (\Phi_3/\Phi_1)\ (1/\Delta V_m) \tag{5b}$$

Since the value of Φ_2 can be calculated from the known values of K_1, K_2, K_3, and K_4 for any given free Ca^{2+} concentration, it can be shown that

$$(\text{slope}/\Phi_2) = (K_e\ K_{m6}/K_{m1})/\Phi_1$$

$$\text{or } \log\ (\Phi_2/\text{slope}) = 4 \log C - \log K_1'\ K_2'\ K_3'\ K_4'\ K_e\ (K_{m6}/K_{m1}) \tag{6}$$

Thus, if all four Ca^{2+} sites must be filled in order for CM to activate PDE, then a plot of log (Φ_2/slope) vs log Ca^{2+} should yield a slope of 4. Furthermore, from the intercept at log $Ca^{2+} = 0$ of such a plot, the size of $K_1'\ K_2'\ K_3'\ K_4'\ K_e$ can be estimated and verified by the equilibrium relationship (cf. Scheme I):

$$K_1'\ K_2'\ K_3'\ K_4'\ K_e\ (K_{m6}/K_{m1}) = K_1\ K_2\ K_3\ K_4\ K_a\ (K_{m6}/K_{m1})$$
$$= K_1\ K_2\ K_3\ K_4\ K_a'$$

Initial rate measurements at pH 7.0, 25°C, in Ca^{2+}-EGTA buffer over a 500-fold variation of CM concentrations and a 10-fold variation of Ca^{2+} concentrations were performed. The results, when plotted in double reciprocal plots, yielded a family of linear lines (each representing a given Ca^{2+} concentration) which appears to interesect on the ordinate. This indicates that K_e is large relative to the concentrations of CM (up to 10^{-6} M) used in these experiments and that $\Phi_3 \simeq \Phi_1$.

When the log (Φ_2/slope) vs log Ca^{2+} plot was made, a slope of 4.03 ± 0.15 was obtained over the Ca^{2+} concentration range of 2.5×10^{-7}-2.5×10^{-6} M. The intercept obtained at log $Ca^{2+} = 0$ was 30.5, which agreed with the value calculated from $-\log K_1 K_2 K_3 K_4 K_a' = 29.7$ very well (within 2.7%). The analysis strongly suggests that all four Ca^{2+} must be bound before CM can activate PDE. Alternatively, it may reflect a cooperativity among the four Ca^{2+} sites that is practically infinitely high. At any rate, the fully-liganded $PDE \cdot CM \cdot Ca_4^{2+}$ species is the dominant form of the activated enzyme.

Can one rule out that the unsaturated forms, say $PDE \cdot CM \cdot Ca_3^{2+}$, are not activated? If one assumes that both the $PDE \cdot CM \cdot Ca_3^{2+}$ and $PDE \cdot CM \cdot Ca_4^{2+}$ forms are fully activated and both have the same K_m, then one can obtain the following expression for the slope of the log (Φ_2/slope) vs log Ca^{2+} plot:

$$\frac{3 + 4\ Ca^{2+}/K_4'}{1 + C^{2+}/K_4'} \qquad (7)$$

It can be seen from Equation 7 that, if K_4' and the free Ca^{2+} concentration are of the same order of magnitude, a slope of 3.5 instead of 4 will be obtained. If the free Ca^{2+} concentration is about 10 K_4', however, the slope value will be 3.9. Since we observed a slope of 4 at Ca^{2+} concentrations as low as 2.5×10^{-7} M, K_4' would have to be $\leq 10^{-8}$ M to give rise to this situation. This would still mean that ($PDE \cdot CM \cdot Ca_4^{2+}$: $PDE \cdot CM \cdot Ca_3^{2+}$) > 10; i.e., the fully liganded species would be the dominant form. The values of K_e, K_1', K_2', K_3', and K_4' cannot be evaluated until kinetic data collected at $[Ca^{2+}] \leq 10^{-8}$ M are available. But it is possible to estimate an upper limit for K_e, the dissociation constant for PDE and CM in the absence of Ca^{2+}. Our data indicate that even at a free Ca^{2+} concentration of 5×10^{-7}, the y intercept in the $1/\Delta v$ vs $1/CM_o$ plot is the same as $1/\Delta V_m$, suggesting that $\Phi_3 \simeq \Phi_1$. This further suggests that $K_1' K_2' K_3' K_4' \leq 10^{-26}\ M^4$ because $C^4/K_1' K_2' K_3' K_4'$ must be > 1. From the known value of $K_e K_1' K_2' K_3' K_4' (K_{m6}/K_{m1}) = 3.2 \times 10^{-31}\ M^5$ and $K_{m6}/K_{m1} = 5$, one can calculate an upper limit for $K_e \gtrsim 10^{-5}$ M. This estimate is consistent with our molecular weight measurement by the airfuge technique. No complex formation between PDE and CM was detected at protein concentrations on the order of 10^{-6} M.

When percent activation of PDE is plotted against log Ca^{2+}, it is found that the Ca^{2+} concentration for 50% activation decreases with increasing CM_o. For example, at $CM_o = 2.09 \times 10^{-8}$ M, $Ca_{0.5}^{2+} = 2 \times 10^{-6}$ M, and at $CM_o = 1.16 \times 10^{-5}$ M, $Ca_{0.5}^{2+} = 2 \times 10^{-7}$ M. The implication is that depending on on the levels of CM, the Ca^{2+} concentration needed for PDE activation in different tissues may be quite different. In this regard, it is also interesting to note that the transformation of chicken embryo fibroblasts by Rom Sarcome virus is accompanied by a marked increase in the cellular concentration of CM. Another implication is that, at normal cellular Ca^{2+} concentration ($< 10^{-7}$), PDE and CM do not form a complex, since the highest known total concentration of CM is about 10^{-5} M (in bovine brain). In other words, the distribution of CM among its various target enzymes may be reshuffled for each Ca^{2+} surge, depending on the cellular levels of the enzymes' substrates and other ions (e.g., Mg^{2+}).

The activation curves described above also reveal that 0-100% activation of PDE is accomplished by a 10-fold increase in Ca^{2+} concentration for a given CM_o. This sharp increase cannot be accounted for by considering $PDE \cdot CM \cdot Ca_4^{2+}$ being the sole activated species without any cooperativity in Ca^{2+} binding (100-fold increase in Ca^{2+} concentration would be required). Therefore, the binding of Ca^{2+} to the PDE·CM complex must involve positive cooperativity, and the existence of four Ca^{2+} binding sites on CM provides an effective on-off switch to activate or deactivate PDE over a narrow range of Ca^{2+} concentration.

The very tight binding of $CM \cdot CA_4^{2+}$ to PDE ($K_a' = 10^{-9}$-10^{-10} M) also points to the importance of having four Ca^{2+} sites on CM. The magnitude of K_a' suggests that PDE is fully activated at a free CM concentration as low as 10^{-8} M. The tight binding, therefore, allows CM to have the capacity of being fully utilized during a surge of Ca^{2+}. The 10^4-10^5-fold increase in affinity between PDE and CM (from $K_e \geq 10^{-5}$ M to $K_a' = 10^{-9}$-10^{-10} M) can be achieved relatively easily through a ~ 20-fold increase in Ca^{2+} affinity for each of the four binding steps. If there was only one Ca^{2+} site on CM, not only will the on-off switch be rendered inefficient, but also the dissociation constant for the $PDE \cdot CM \cdot Ca^{2+}$ complex will have to be lowered to $\leq 10^{-10}$-10^{-11} M. Such a low dissociation constant for Ca^{2+} will further reduce the efficiency of the control process in terms of time. Thirty minutes or more may be needed to completely deactivate PDE because of the slow off-rate constant (~ 10^{-3} s^{-1}) of Ca^{2+}.

The model described in Scheme I should be applicable to other CM-regulated enzyme systems. It may be a general mechanism by which various enzymes are activated by Ca^{2+} through the mediation of CM.

Proposed Course of Research

(1) Interaction of calmodulin and its binding protein with the cyclic nucleotide phosphodiesterase.

(2) Properties and mechanism of action of the high molecular weight (80,000) calmodulin-like protein.

(3) Effect of Ca^{2+} and phosphorylation on the calmodulin-regulated protein kinases in the glycogen phosphorylase and the tryptophan hydroxylase systems.

Publications

Milman, H.A., Cooney, D.A., and Huang, C.Y.: Studies on the Mechanism of the Glutamine-Dependent Reaction Catalyzed by Asparagine Synthetase from Mouse Pancreas. J. Biol. Chem. 255: 1862-1866, 1980.

Bale, J.R., Chock, P.B., and Huang, C.Y.: The Nature of Negative Cooperativity in Alkaline Phosphatase: Kinetic Patterns Contrary to the Flip-Flop Model. J. Biol. Chem. 255, 1980, in press.

Bale, J.R., Huang, C.Y., and Chock, P.B.: Transient Kinetic Analysis of the Catalytic Cycle of Alkaline Phosphatase. J. Biol. Chem. 255, 1980, in press.

Wang, J.H., Sharma, R.K., Huang, C.Y., Chau, V., and Chock, P.B.: On the Mechanism of Activation of Cyclic Nucleotide Phosphodiesterase by Calmodulin. Ann. N.Y. Acad. Sci., 1981, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00225-03-LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Inactivation of Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Rodney L. Levine	Senior Surgeon, U.S.P.H.S.	LB	NHLBI
Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Suspensions of cells of Klebsiella aerogenes inactivate and then proteolytically degrade glutamine synthetase. The inactivation reaction bears characteristics similar to cytochrome P-450 dependent reactions. Ascorbic acid mimics some P-450 reactions, and ascorbate does inactivate glutamine synthetase.

Ascorbate-inactivated glutamine synthetase was studied to identify the altered amino acid(s). Spectral studies were consistent with the oxidation of one tyrosine/subunit to a dopa-like compound. Alkaline hydrolysis documented that the 2 tryptophan/subunit were not altered. Acid hydrolyses suggest a loss of one tyrosine and one histidine per subunit.

The chemical changes identified so far can be mediated by cytochrome P-450 dependent systems. Thus, such systems may be physiologically involved in the degradation of specific proteins.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Suspensions of cells of Klebsiella aerogenes inactivate and then degrade glutamine synthetase. Ascorbic acid also inactivates glutamine synthetase. This reaction provides a model of the inactivation step which occurs within cells. The objectives of this project are determination of the chemical changes which occur on inactivation of glutamine synthetase; purification and characterization of the inactivating and proteolyzing components of the cell; and assessment of the physiologic control of inactivation and proteolysis.

Major Findings

This year's efforts were concentrated on determining the chemical changes which occur upon oxidative inactivation of glutamine synthetase.

1. Tryptophan is not altered.

The second derivative of the absorption spectrum was unchanged by inactivation in the region specific for tryptophan. Similarly, with excitation at 300 nm, the fluorescence emission spectrum was unchanged. Finally, alkaline hydrolysis of the protein demonstrated that the 2 tryptophan/subunit were unchanged.

2. Spectral changes suggest oxidation of a tyrosine.

Upon inactivation, the absorptivity of glutamine synthetase increases. The difference is small, 5% at the peak, but real. It occurs on inactivation of either unadenylylated or adenlylyated enzyme.

Construction of a division spectrum showed that the difference spectrum was not an artifact of differences in concentration of the inactivated and control enzymes. (In working with small spectral changes, matching of protein concentrations is critical but difficult.) Dividing one spectrum by another will yield a flat line if the two spectra are of the same substance at different concentrations. If the spectra differ in other than concentrations, then the division spectrum will not be flat.

The observed difference spectrum suggested oxidation of an aromatic amino acid. Specifically, the difference spectrum was qualitatively and quantitatively consistent with the oxidation of a single tyrosine residue to a dopa-like residue. Computer-simulated synthesis of a glutamine synthetase molecule revealed that substitution of one tyrosine for dopa yielded spectral changes similar to those on inactivation by ascorbate.

3. Amino acid analyses.

Amino acid analysis of the inactivated enzyme have been compared to that of the control. These studies have proven difficult to reproduce because they require detection of changes on the order of 5%. Modifications of techniques may yield the required precision, but for now results are tentative: The inactivated enzyme has lost one of 16-17 histidines/subunit and one of 16 tyrosines/subunit. A new peak was detected in the acidic amino acid region, but it has not yet been identified.

Dopa has not been detected either on the analyzer or by fluorimetric assay. However, control experiments demonstrated instability of dopa under hydrolysis conditions with hydrochloric or methanesulfonic acids.

4. Inactivated enzyme is more susceptible to degradation.

We noted earlier that in cell suspensions, inactivation preceeds degradation of glutamine synthetase. We hypothesized that the modifications associated with inactivation might "mark" the protein for subsequent degradation. Experiments carried out by C. N. Oliver and E. R. Stadtman documented that ascorbate-inactivated enzyme was much more susceptible than the control to proteolysis by an extract from *Escherichia coli*.

5. Analytical Techniques.

Studies on the chemical changes associated with inactivation of glutamine synthetase led to the development of 2 new methods of analysis: 1) quantitation of aromatic amino acids within proteins by second derivative spectroscopy and 2) rapid alkaline hydrolysis of proteins for tryptophan determination.

The first method now permits quantitation of phenylalanine, tyrosine, and tryptophan in proteins denatured in guanidine hydrochloride. The method requires about 1 minute and gives results with less than 4% error in each amino acid. The success of the technique depended on development of models of the amino acids which mimic the spectral characteristics of the residues within proteins.

As an incidental finding, the method revealed that glutamine synthetase contains 2 tryptophan/subunit. Earlier studies using direct UV methods gave a value of 3. Alkaline hydrolysis confirmed that the correct value is 2.

The second method, for alkaline hydrolysis, permits determination of the tryptophan content of a protein within several hours. Sub-nanomole amounts suffice for the assay.

Significance to Biomedical Research

The net concentration of any protein represents the balance point of its synthesis and degradation. Although the biochemical mechanisms of protein synthesis are known in detail, very little is known of the pathways of protein degradation.

In previous reports, we noted that inactivation of glutamine synthetase by extracts of Klebsiella shared characteristics of the cytochrome P-450 oxidase systems. Since ascorbate can mimic some P-450 dependent reactions, ascorbate was tested. Ascorbate did inactivate glutamine synthetase. Studies summarized in this report suggest that inactivation is associated with oxidation of a tyrosine residue to a dopa-like compound. Such a reaction could well be catalyzed by a P-450 dependent system in vivo.

Studies by C. N. Oliver and E. R. Stadtman in this laboratory have now demonstrated that ascorbate-inactivated glutamine synthetase is more susceptible than control enzyme to proteolytic degradation. Also, a purified mammalian P-450 system mediates inactivation and degradation of bacterial glutamine synthetase. We therefore hypothesize that cytochrome P-450 may function physiologically in the degradation of proteins.

Knowledge of the mechanism of degradation of specific proteins would be valuable. Degradation of specific proteins is of paramount physiologic importance during growth and development of the fetus and child. Abnormal protein degradation occurs in several disease states - - muscular dystrophy is perhaps the best known.

Proposed Course of Research

Studies on the chemical changes in inactivated glutamine synthetase will continue. Studies on cytochrome P-450 systems from mammalian and bacterial sources will receive increasing emphasis.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00226-03 LB

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October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Selenoproteins: Synthesis and Role of Selenium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Gregory Lee Dilworth	Staff Fellow	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.0	OTHER: 0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clostridium barkeri incorporates selenium into the enzyme nicotinic acid hydroxylase as shown by copurification using numerous protein separation techniques. Nicotinic acid hydroxylase, like many flavoproteins, possesses NADPH oxidase activity. The activity when nicotinic acid serves as the substrate is more sensitive than the NADPH oxidase to reduction and alkylation, treatments that frequently modify selenium compounds. This suggests that the selenium is associated with the nicotinic acid-reactive site on the enzyme.

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Project Description

Objectives: To elucidate the role of selenium in selenoproteins and to determine the biosynthetic pathway of their synthesis.

Progress

(1) Attempts to stabilize the very labile nicotinic acid hydroxylase activity with numerous reagents have failed. However, it has been possible to purify the enzyme to greater than 80% purity using several very rapid separation procedures and still retain a substantial amount of activity. Selenium remains associated with the enzyme activity during these procedures and during analytical disc gel electrophoresis.

(2) Nicotinic acid hydroxylase, like many flavoproteins, has an associated activity, NADPH oxidase. The hydroxylase activity of the enzyme is more labile than the oxidase activity to alkylation and reduction. The known sensitivity of organoselenium compounds to these treatments suggests that the selenium may be located near or at the hydroxylation site.

(3) Structural studies on the chemical form of the selenium moiety in nicotinic acid hydroxylase have been hampered by the inability to get purified protein which contains more than 1% of active enzyme. The rapid purification procedure mentioned in Section 1 should permit the study of the nature of the selenium in the active enzyme.

Publications

1) J. B. Jones, G. L. Dilworth and T. C. Stadtman. Identification of Selenocysteine in the Selenium-dependent Formate Dehydrogenase of *Methanococcus vannielii*. Arch. Biochem. Biophys. 195: 255-260, 1979.

2) Bacterial Selenoproteins. T. C. Stadtman, G. L. Dilworth and C.-S. Chen. 3rd Int. Symp. on Selenium and Tellurium Chemistry, in press.

3) Selenium-Containing Nicotinic Acid Hydroxylase from *Clostridium barkeri.* Gregory L. Dilworth. 3rd International Symposium on Selenium in Biology and Medicine, in press.

4) Reconstitution of Mesophilic Microbial Associations which Ferment Cellulose to various Products. G. L. Dilworth, J. Wiegel, L. Ljungdahl and H. D. Peck. International Symposium on Biomass Conversion, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
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PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 00227-03 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Interaction of the Dye Cibacron Blue F_3GA with Glutamine Synthetase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mary Marcia Federici	NIH Postdoctoral Fellow	LB	NHLBI
Other:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

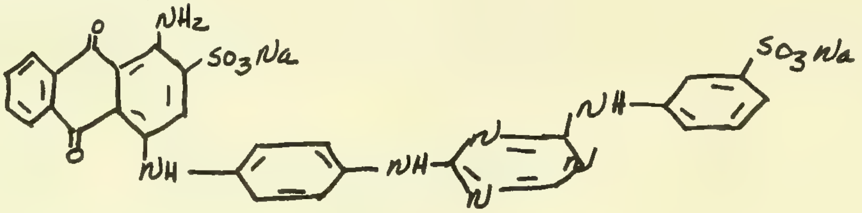
SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this investigation is to extend studies demonstrating the effects of the protein, glutamine synthetase (GS), on the visible spectrum of the dye Cibacron Blue F_3GA. Four chromatographically distinct subfractions of Cibacron Blue have been isolated. The difference spectra obtained when each of these subfractions binds to taut, relaxed, or subunit forms of GS clearly differ from each other and can be displaced by addition of ADP to the dye plus enzyme sample. Incubation of one of the dye subfractions with GS results in an altered difference spectrum with time which can no longer be displaced by ADP. Time-dependent changes in the visible dye spectrum are obtained upon dissociation of the protein in urea with differences seen for $GS_{\overline{2.6}}$ and $GS_{\overline{11}}$. These changes can be correlated with light scattering data obtained under the same conditions. An aromatic spectral perturbation is obtained in the standard urea dissociating system. A method is described for quantitating aromatic amino acid residues in intact denatured proteins using second derivative UV spectroscopy.

PHS-6040
(Rev. 10-76)

Project Description and Background

Previous studies by others have shown that the dye, Cibacron Blue F3GA (structure shown below), can be used for probing the nucleotide binding sites in certain proteins.



The measurement of dye versus dye plus enzyme spectrum for various kinases and dehydrogenases yields a characteristic difference spectrum with a positive maximum at 660-680 nm. The observed difference spectrum is lost upon displacing the dye from the nucleotide binding site by the addition of ADP to the dye plus protein sample.

Escherichia coli glutamine synthetase is a multisubunit enzyme which requires the nucleotide, ATP, in its catalytic formation of glutamine from glutamic acid and NH_3. Several structural forms of the protein are known to exist and have been studied in this laboratory. Removal of divalent cations from the native (taut) forms of GS converts the enzyme to a relaxed (catalytically inactive) configuration that is characterized by exposure of sulfhydryl groups and a shift of aromatic amino acid residues from a hydrophobic to a hydrophilic environment. Also, at high pH (> 8.5) or in the presence of urea, the relaxed enzyme is dissociated to subunits of 50,000 molecular weight. It is the scope of this investigation to explore the interaction of Cibracron Blue F3GA with these three known structural forms of glutamine synthetase.

Summary of Major Findings from 1979

The protein, glutamine synthetase, affects the visible spectrum of the dye Cibacron Blue F3GA. A characteristic dye difference spectrum is obtained with the native (taut) form of the protein having a maximum at 640 nm and a minimum at 700 nm. When the dye binds to the relaxed and subunit forms of the protein (two other structural forms of GS), the spectra obtained differ from each other and clearly differ from the characteristic spectrum obtained with the native (taut) GS. Thus, three different structural forms of glutamine synthetase yield distinctly different difference spectra in the presence of Cibacron Blue F3GA. Initially, samples of Cibacron Blue F3GA were obtained from commerical sources and used without purification. However, TLC of the dye in various solvent systems demonstrated at least six (possibly eight) distinct subfractions of the dye present in commercial preparations. Four of the major subfractions of Cibacron Blue F3GA were resolved and purified using preparative silica gel columns.

Current Major Findings 1980

A. Characteristics of Isolated Dye Subfractions. Four major subfractions of the dye were isolated as stated previously using preparative silica columns and an ethyl acetate/1-butanol solvent system. Rechromatography of the isolated fractions demonstrated that each of the four components were chromatographically homogeneous. Estimates of the content of each subfraction in the crude dye mixture can be obtained from the yields obtained from the silica gel columns.

$K_D \times 10^{-7}$	Fraction	% Total	Rf [1]
1.4	I	8 [2]	0.84 ± .01
1.64	II	- [3]	0.80 ± .03
1.9	III	35 [2]	0.75 ± .02
2.6	IV	57 [2]	0.71 ± .03

[1] Solvent system = THF/H_2O 48:7. Mixing experiments with the above fractions confirmed that they are indeed chromatographically separate and nearly homogeneous entities with no further species formed during mixing or chromatographic separation.

[2] Obtained from Ciba Geigy.

[3] Present in trace amounts in samples obtained from Ciba Geigy; present and isolated in larger quantities from Pierce Chemical Company dye samples.

The difference spectra obtained when each of these subfractions binds to taut, relaxed, and subunit forms of GS clearly differ from each other in shape and amplitude, implying differences in their interaction with each of the structural forms of glutamine synthetase. Immediate addition of concentrated ADP-Mn solutions to the dye plus enzyme sample results in a loss of the observed difference spectrum, indicating interaction of the dye at the nucleotide binding site in the protein. From these experiments, a dissociation constant for the GS-dye complex for each respective fraction can be calculated as shown in the table above. However, when further experiments were performed to obtain dissociation constants (e.g., spectral titrations, sedimentation) a good agreement with the above results is not obtained. This can probably be explained by the phenomenon of dye stacking which has been shown to occur. This was demonstrated by studying the concentration difference spectrum of the dye. Using a sample of the dye in a 0.5 cm path length cell and a 1:10 dilution of that sample in 5.0 cm path length cell, results in a difference spectrum implying that a concentration dependent process is occurring. The amplitude of this difference spectrum varies with the concentration of dye used; a sigmoidal shaped curve was generated for this effect with dye concentrations ranging from 8.0 μM to 150 μM. These results suggest that any method used for determination of dissociation constants employing determination of free dye concentrations (e.g., spectral titration) will be affected by the concentration dependent dye stacking which has been observed.

B. Use of the Dye to Monitor Reactions and Structural Changes in GS. Immediate addition of 3.0 mM ADP-Mn to peak fraction I + GS results in nearly a total loss of the observed difference spectrum. However, when serial additions of ADP-Mn are used a point is reached when you can no longer displace the difference spectrum with very high levels of ADP-Mn. This phenomenom was explained by incubation of peak I + GS and scanning the spectra with time. A time-dependent spectral change was obtained in which the maximum was altered from 666 nm to 638 nm with an isobestic point at 646 nm and a t 1/2 of ~ 100 minutes (under conditions of ~ 80% saturation of GS with peak I). After the completion of the reaction (~ 5 hours), addition of 1.7 mM ADP-Mn resulted in no loss of the difference spectrum. Apparently, incubation of this peak fraction with GS results in an altered form of the dye protein complex in which the dye can no longer be displaced by the addition of nucleotide. This is not observed for the other three fractions isolated. It is noteworthy that this dye fraction corresponds chromatographically with a fraction reported by other workers to inactivate phosphoglycerate kinase.

Other time-dependent spectral changes are observed in the visible dye spectrum by dissociating the protein in urea (these changes are slower than those observed by dissociating the relaxed enzyme with high concentrations of base). Incubation of the native protein at pH 7.8 at urea concentrations ranging from 2.9 to 4.0 M in a standard dissociating system (15-20 μM GS subunits, 36 mM Hepes, 100 mM KCl, 100 μM dye, 1 mM $MnCl_2$) results in time-dependent changes at several wavelengths. The rate of the reaction is slower for $E_{\overline{2.6}}$ as compared to $E_{\overline{II}}$. In addition, a considerable lag time is observed for the reaction with $E_{\overline{2.6}}$ and is dependent on urea concentration. However, no lag time in the visible dye spectral changes is observed with $E_{\overline{II}}$ at urea concentrations higher than 3.0 M (where a ~ 40 minute lag time with $E_{\overline{2.6}}$ is obtained).

At an intermediate urea concentration (3.42 M), there is a differential loss of enzyme activity with $E_{\overline{11}}$ losing more activity as a function of time than $E_{\overline{2.6}}$. Inclusion of dye in the incubation results in an increase in the extent of the inactivation for both forms of the protein. Incubation of the protein ($E_{\overline{2.6}}$) in the standard dissociating system minus dye (3.3 M urea) results in time-dependent changes at λ 290.2, 280, and 286 implying that an aromatic perturbation is obtained upon dissociation of GS in urea. However, no lag time is observed in these ultraviolet spectral changes which can be stopped by the addition of $MnCl_2$ or the reaction driven to completion by the addition of EDTA. Light scattering in the standard system shows a slow exponential decrease in scattering which can be correlated with changes in the visible dye spectrum under the same conditions. No reaggregation was observed during the course of the light scattering experiments. Taken together, the light scattering experiments and the loss of activity, both indicate that the GS dodecamer is dissociating under the standard conditions used. This dissociation is characterized by an aromatic perturbation and can be monitored by changes in the visible dye spectrum of Cibacron Blue F3GA.

No conclusive explanation has been obtained as yet for the lag time observed in the dye mediated dissociation reaction.

C. Further Notes on Purification of Cibacron Blue F_3GA. Elemental analysis of peak fractions II and III resulted in considerable discrepancy as compared to the calculated values (for C, H, and N). This indicates that although chromatographically homogeneous these fractions are not analytically pure. Determination of the molar extinction coefficient for fraction IV results in a value of 12.3 mM^{-1}. Further purification of fraction III by passage over Dowex 50 followed by titration with NaOH (3 equivalents of acid groups titrated/mole) resulted in a value for E = 11.43 mM^{-1} for this dye fraction (literature value for unfractionated dye E = 13.6 mM^{-1}). Further attempts at purification of the dye in reverse phase C_{18} hydrophobic HPLC systems using various solvent systems (pH 8.0 ammonium bicarbonate-MEOH; H_2O-MeOH; ion pairing pic A reagent-MeOH) resulted in no improvements in the resolution of the unfractionated dye as compared to silica chromatography. These results indicate that it is perhaps the ionic properties of the dye fractions which dictate their chromatographic behavior and separation.

Proposed Course of Action

1. Further investigation of the dye mediated dissociation of glutamine synthetase in urea with particular emphasis on determining what process is responsible for the lag time observed.

2. Use of one of the major subfractions of the dye to monitor the dissociation of the protein as compared to the presently reported studies with unfractionated dye.

3. Further characterization of the time-dependent spectral change observed by incubation of fraction I with glutamine synthetase including studies of effects on enzyme activity followed by exhaustive dialysis and gel electrophoresis in denaturing systems. Investigate the possibility that this is a photochemical reaction.

4. Investigate the influence of metabolic effectors on dye mediated spectral changes for the various reactions described.

5. Further characterization of the chemical differences between the dye fractions isolated.

Collaborative Work

I have collaborated with Dr. R. L. Levine developing a method for quantitating aromatic amino acids in denatured proteins. This work is based on the observation that the ultraviolet absorption proteins for all proteins can be accounted for by the three aromatic amino acids (tyrosine, tryptophan, and phenylalanine) plus contributions from cystine and light scattering. Quantitation of these residues from the spectrum of the intact protein was achieved using second derivative UV spectroscopy (which eliminates interferences from cystine and light scattering). This treatment aids in the partitioning of the protein spectrum into each of the separate components and the heights in derivative spectra are proportional to concentration. In addition, a microprocessor assisted spectrophotometer was used which computes the relative concentration for each compound present in a multicomponent mixture by comparison to known standards.

The accuracy of the method requires that the spectra of the standards used match the spectra for the corresponding residues within the protein. The N-acetyl ethyl esters of the amino acids are commonly used as standards. This is adequate for phenylalanine as judged by comparison to rabbit parvalbumin (contains 9 Phe and no Tyr or Trp). However, the spectra of tyrosine and tryptophan residues in proteins denatured in 6 M guanidine HCl are slightly red shifted compared to model compounds in the same solvent. Solvent perturbation of the tryosine derivatives by 55% methanol improves them as standards (judged by a comparison to a protein with no Trp). The spectrum of tryptophan from mellitin (contains no Phe or Tyr) was used as a standard for this amino acid. Analytical results were obtained using the following standards: N-acetyl phenylalanine ethyl ester in 6 M guanidine HCl; N-acetyl tryosine ethyl ester in 55% MeOH; spectrum of tryptophan from mellitin. Results obtained for a number of sequenced proteins are within 4% error of the expected values even for a heme containing protein.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00228-03 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Purification of an Enzyme of the First Cycle in the Covalent Modification Cascade of Glutamine Synthetase of Escherichia coli (title change)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Emilio Garcia	Staff Fellow	LB NHLBI
Others:	Sue Goo Rhee	Research Chemist	LB NHLBI
	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have used Escherichia coli strains, which carry the gene encoding the uridylyltransferase enzyme in a ColEl hybrid plasmid, to obtain elevated amounts of this protein. Strain JA200/pLC 38-39, previously shown to produce uridylyltransferase (UT) and uridylyl-removing (UR) enzymes at levels 25-fold those found in the wild type strain, was used to purify these two activities to homogeneity. Copurification of these two activities throughout the procedure indicated that the UT·UR enzyme is bifunctional in nature. Molecular weight determination in polyacrylamide gels showed UT·UR to be 110,000 in size. The enzyme is composed of a single subunit. Results from gel filtration and gel electrophoresis steps indicate that the protein oligomerizes readily.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The immediate objective of this project is the purification and characterization of the uridylyltransferase enzyme of the covalent modification cascade of glutamine synthetase in Escherichia coli. The long-range objective remains the detailed biochemical characterization of the components of this cascade system. Such characterization requires obtaining significant amounts of the four proteins involved (e.g., P_{II} regulatory protein, adenylyltransferase, and UT·UR) in purified form. The present purification of UT·UR may enable, for the first time, the in vitro study of the interaction among all these proteins, their effectors, and their action on the adenylylation-deadenylylation of glutamine synthetase.

Major Findings

A. Purification of UT·UR. The UT·UR enzyme has been purified utilizing the following procedure: (1) use of a plasmid-containing strain which overproduces the enzyme giving an effective purification of 25-fold; (2) a polyethylene glycol precipitation; (3) chromatography on DEAE-cellulose; (4) a Matrix Blue gel A column; and (5) separation in a sephacryl-300 column. The last step gives rise to homogeneous protein. Due to the very unstable nature of UT·UR, great variability is obtained in calculating recovery for these steps. Thus, it is not really meaningful to express the exact fold purification, or to attempt to give an accurate percent recovery for the above procedure. However, the approximate purification of UT·UR obtained by these steps is about 5,000-fold.

For purification of UT·UR, E. coli cells were grown on a minimal medium (Vogel-Bonner) with 0.4% glucose as carbon source and with ammonia (17 mM) as the nitrogen source. The medium was supplemented with 0.3 mM each of tryptophan, threonine, and leucine to satisfy the amino acid requirement of this strain. During efforts to optimize the yield of cells grown for this purification, it was found that although the specific activity of UT·UR on cells grown in rich medium (medium supplemented with 1% yeast extract) is identical to that of cells grown on minimal medium, the rich medium grown cells yielded preparations of UT·UR that cannot be successfully purified by the above procedure. The significance of this finding has not been pursued.

B. Preliminary Characterization of UT·UR Enzyme. Studies of the post-sephacryl-300 fraction in 4 to 20% gradient native polyacrylamide gels showed a single protein band which migrates at a position corresponding to a molecular weight of 100,000 to 110,000.

SDS polyacrylamide gels at this same homogeneous fraction showed a single band which migrates at a position corresponding to a molecular weight of 100,000. Since this molecular weight is essentially the same as that obtained on native gels, it showed that UT is composed of a single monomer of 100,000 molecular weight.

Previous genetic evidence had suggested that the uridylyltransferase and uridylyl-removing activities may be the action of a single enzyme or enzymic complex. Mutant strains in E. coli, Salmonella, and Klebsiella with a lesion in the glnD locus have always been found to lose both activities simultaneously, and the revertants of these mutants always regain both activities simultaneously. However, since there has been conflicting biochemical evidence (see Francis and Engleman (1978) Arch. Biochem. Biophys. 191:590-601) which reported differential inactivation of UT and UR, the existence of two separate activities in a single polypeptide has never been conclusive.

Assays of the uridylyl-removing activity at each step of the purification of UT has shown that the two activities copurify. Furthermore, the known inhibition of UR activity by CMP remains constant throughout the purification. These results estabished conclusively that a single polypeptide possesses both UT and UR activity.

Proposed Course of Action

Studies on the effects of different metabolism on the UT·UR enzyme will be carried out using the purified UT·UR (see reference). New steps such as a newly reported affinity column in which the α-ketoglutarate analogue 3-(R,S)-3-bromo-2-ketoglutarate is linked to an agarose column [Hartman, F. C., Fed. Proc. 39, 1980) will be tried in order to facilitate the purification and/or increase the yields of UT·UR obtained during our purification.

The effects of α-ketoglutarate and glutamine on both the uridylylation and deuridylylation reaction of UT·UR will be examined directly. These two metabolites are known to affect the adenylylation-deadenylylation reaction of ATase (the second cycle of the cascade), but the effects on the first cycle (uridylylation-deuridylylation) have never been directly measured.

Studies on the stabilization of UT·UR will be carried out subsequently. The enzyme is very unstable at low protein concentrations. There is a possibility that addition of some purified P_{II} protein or other pure protein, easily separable from UT·UR may stabilize UT·UR and/or inhibit its polymerization. When enough quantitation of UT·UR is obtained, AA analysis of the enzyme will be carried out, and Ab against the protein will be used. This will facilitate stricter quantitation of the levels of UT·UR present in the cell, and it will help in the analysis of mutants lacking components of the covalent modification of glutamine synthetase.

Publications

Garcia, E., Federici, M.M., Rhee, S.G., and Berberich, M.A.: Glutamine Synthetase Cascade: Enrichment of Uridylyltransferase in *Escherichia coli* Carrying Hybrid ColEl Plasmids. Arch. Biochem. Biophys. 203, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00229-02 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of Adenylylation of Glutamine Synthetase in Permeabilized E. coli Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Umberto Mura	Visiting Fellow	LB NHLBI
Other:	Earl R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)

None

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Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.1	OTHER: 0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To study the cascade mechanism control of glutamine synthetase (GS) under conditions that well approximate the in vivo situation, Escherichia coli cells have been made permeable to only small molecules.

The GS activity in permeabilized cells (but not in untreated cells) can be measured in situ by means of the same procedure used for measuring activity of the soluble enzyme. In addition to GS, the permeabilized cells retain all the protein components involved in modification of GS and therefore are able to catalyze the adenylylation and deadenylylation of GS in situ.

This interconversion responds to alterations in concentration of ATP, UTP, Pi, Mg^{++} and allosteric effectors (α-ketoglutarate and glutamine) as predicted by the earlier in vitro studies with cell-free systems.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Escherichia coli glutamine synthetase is regulated by the covalent attachment and removal of 5'-AMP residues from each of the enzyme's 12 subunits. This adenylylation and deadenylylation process which has been extensively studied in vitro is regulated by a complex cascade mechanism control.

The primary objective of this project, as stated in the 1979 Annual Report, is to study the process under conditions closely resembling the physiological state of the cell. The approach described here, making use of permeabilized E. coli cells, has shown it to be a useful means of studying the GS regulatory system in situ.

Major Findings

A. Permeabilization of E. coli cells. The preferential permeabilization of E. coli cells to small molecules has been obtained by treating the cells with Lubrol WX after a freeze-thaw cycle. The freezing is essential to obtain permeabilization, and stationary phase E. coli W was frozen as a pellet in liquid nitrogen and thawed when used. The time course of permeabilization has been followed at 37°C at different detergent concentrations. Both the rate and the final extent of permeabilization are not significantly affected by using more than 1 mg/ml of Lubrol.

The routine permeabilization conditions which give good reproducibility in the permeabilization of cells are: cells at a final $O.D._{650} = 40$, 1 mg/ml Lubrol WX, and 10 mM $MgCl_2$ in 50 mM 2-methylimidazole buffer (pH 7.2) containing 1 mM of 2-mercaptoethanol for 90 minutes at 37°C. Under these conditions, between 80-90% of the total GS activity can be detected directly on the permeabilized cells.

The presence of 10 mM $MgCl_2$ during permeabilization apparently reduces the extent of nucleic acid catabolism without significant influence on the permeability.

The Lubrol treatment does not affect the GS activity which can be recovered on the permeabilized cells almost completely. Less than 5% of the GS activity is detectable on the permeabilizing medium after treatment with the detergent, and no significant leakage occurs by keeping the permeabilized cells in buffer at 0-4°C for 4 hours. Less than 6% of several enzymes (glutamate dehydrogenase, purine nucleoside phosphorylase, adenosine phosphorylase, thioredoxin) tested is released into the suspending buffer during the permeabilization. The γ-glutamyltransferase activity in permeabilized cells shows the same dependency on the concentration of ADP (in the suspending buffer) as does the enzyme in crude homogenates.

B. Adenylylation and Deadenylylation of Glutamine Synthetase. The state of GS adenylylation in permeabilized cells can be varied by suspending the cells in buffer containing various concentrations of metabolites and divalent cations that are known to affect the activity of purified adenylyltransferase.

The adenylylation-deadenylylation reactions can be arrested by cooling the cell suspension to 0°C, and can be permanently stopped either by sonication at 0 to 4°C, or by inhibition of the adenylyltransferase by incubation with 0.5 mg/ml of cetyltrimethylammonium bromide (CTAB) for 30 minutes at 0°C. These findings allow manipulation of the cells after treatment with substrates and effectors of the GS cascade mechanism control, and thus measurement *in situ* of the GS state of adenylylation. The steady-state level of adenylylation of GS in permeabilized cells of *E. coli* W grown in 5 mM glutamine as the sole nitrogen source responds to variations in the extracellular concentration of α-ketoglutarate and glutamine as predicted by the theoretical analysis and the *in vitro* results of a monocyclic cascade system (Stadtman, E.R. and Chock, P.B. (1978) Curr. Topics Cell. Regul. 13, 53-93; Rhee, S.G., Park, R., Chock, P.B., and Stadtman, E.R. (1978) Proc. Natl. Acad. Sci. USA 75, 3138-3142).

The level of the substrates of the adenylyltransferase, ATP, and Pi also affect the steady-state level of the GS adenylylation. The role of ATP as allosteric effector in the deadenylylation reaction which was reported earlier in the *in vitro* system has been observed in permeabilized cells after ATP depletion by arsenate.

The state of adenylylation of GS in permeabilized cells is strongly affected by the Mg^{++} concentration. Under both adenylylating and deadenylylating conditions, the metal ion is required to modify the adenylylation level of GS.

The deadenylylation of GS in permeabilized cells grown in 10 mM glutamine as the sole nitrogen source is activated by UTP. Since the P_{II} protein in cells grown in 10 mM glutamine would be expected to be in the P_{IIA} form (deuridylylated), the UTP dependence of deadenylylation probably represents the uridylylation of P_{II} by UTase.

While the activation by UTP persists after removal of UTP when cells are subsequently manipulated at pH 7.2, the incubation of the same UTP activated cells at pH 8.6 with proper effectors, deactivates the system and undergoes the deadenylylation process again dependent on UTP.

These findings are consistent with a previous report (Francis, S.F. and Englemann, E.G. (1978) Arch. Biochem. Biophys. 191, 602-612), since pH 8.6 is the optimum pH for the UR activity and a condition in which the inhibition by both pyrimidine and purine mononucleotides are 10 times lower than at pH 7.6.

Escherichia coli cells, grown in 5 mM glutamine as the sole nitrogen source, in which the starting GS level of adenylylation is relatively low ($\bar{n} = 3$), are unaffected by the UTP treatment. The deadenylylation reaction in these cells occurs, in fact, in both the absence and presence of UTP, and for reasons not yet understood, this happens also after treatment favoring the deuridylylation of the P_{II} protein.

Proposed Course of Research

The different responses obtained from the cells grown in 5 and 10 mM glutamine as the sole nitrogen source to the UTP treatment is at present the only result which cannot easily be explained on the basis of the *in vitro* studies.

The immediate objective of this research is to explain these results. Several hypothesis will be tested (*e.g.*, the UTase-UR system is destroyed during the permeabilization of the 5 mM glutamine grown cells; either an inhibitor of the UR activity is present, or an activator is involved in these cells).

Moreover, the conditions to better control the UTP-dependent deadenylylation in permeabilized cells grown in 10 mM glutamine as the sole nitrogen source will be studied. The results should give more information on the functioning *in situ* of the first cycle of the GS cascade control mechanism.

Publications

Stadtman, E.R., Mura, U., Chock, P.B., and Rhee, S.G.: The interconvertible enzyme cascade that regulates GS activity. In Palacios, R., Meister, A., Cohen, P.P., (Eds.): Proceedings of the Meeting on Glutamine: Metabolism, Enzymology, and Regulation, Mexico City, 1979, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00231-02 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Development of a Pure In vitro Interconvertible Enzyme Cascade System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Emily Shacter Noiman	Biochemist (Guest Worker)	LB NHLBI
Others:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	P. B. Chock	Research Chemist	LB NHLBI
	S. G. Rhee	Research Chemist	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two enzymes, a cyclic-AMP-dependent protein kinase and a phosphoprotein phosphatase, have been purified to near homogeneity from bovine heart. A suitable substrate has been sought so that the kinetics of a monocyclic cascade system can be defined and understood. Three potential substrates have been purified for this purpose. Histone H1 was purified to homogeneity, but was found to activate the protein kinase. Attempts to modify the H1 and eliminate this activation effect were only partially successful. Consequently, the use of H1 had to be abandoned. A different protein with one phosphorylatable site is liver pyruvate kinase. This enzyme was purified from beef liver, but could not be phosphorylated; the reason for this is not yet clear. Human erythrocyte pyruvate kinase was also partially purified as a potential substrate for the phosphorylation-dephosphorylation cascade. While this work was in progress, a novel substrate was discovered; the 20,000 dalton light chain of chicken gizzard myosin. In order to assess the applicability of this to a model cascade system, and because it may be important in the regulation of smooth muscle contraction, the protein chemistry and kinetics of myosin light chain phosphorylation by protein kinase were characterized.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: To synthesize a well defined *in vitro* cyclic cascade system which can be used to test the kinetic equations and predictions, which have been developed in this laboratory, describing such intracellular systems. Phosphorylation-dephosphorylation of a protein substrate has been chosen as a model cascade system.

Major Findings

It was found that the 20,000 Dalton myosin light chain has one phosphorylatable site identical to that phosphorylated by myosin light chain kinase, and that the protein was a suitable substrate for both the cAMP-dependent protein kinase and phosphoprotein phosphatase previously purified. These results are currently being prepared for publication.

Proposed Course of Action

Now that a suitable substrate for the protein kinase and phosphoprotein phosphatase has been found, experiments will be carried out to quantify the kinetic variables and test the equations used to define a steady-state condition.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00233-02 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Menadione-Dependent p-nitrophenyl-phosphatase of Clostridium sticklandii

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J.N. Davis	Research Assistant	LB NHLBI
Other:	T. C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	0.1	1.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A quinone-dependent phosphatase was previously purified to homogeneity from Clostridium sticklandii. The recent observations that ^{32}P could be cleaved from both [^{32}P] phosphocasein and [^{32}P] phosphohistone suggest that the natural substrate of this enzyme is a bacterial phosphoprotein. After selective removal of the phosphatase from crude C. sticklandii extracts by passage over a quinone affinity column, the resulting preparations were labeled with ^{32}P and surveyed as substrate for the phosphatase.

PHS-6040
(Rev. 10-76)

Project Description

1. To isolate and identify the natural substrate of the bacterial quinone dependent p-nitrophenylphosphatase.

2. Test the ability of the purified phosphatase to interact with the glycine reductase system and serve as alternate acceptor for the phosphate that is esterified concomitant with glycine reduction. In the *in vito* system with ADP as the phosphate acceptor, ATP is formed. If direct interaction with the phosphatase occurs it would suggest that the phosphatase normally acts as a phosphate carrier in some cases such as an energy-dependent membrane transport system, a regulatory system involving protein phosphorylation and dephosphorylation, etc. In such a system a protein bound phosphate ester might be required as substrate rather than ATP.

Major Findings

(1) Preparations of [^{32}P] Histone type II As from calf thymus which had been phosphorylated with [γ^{32}P] ATP by Rabbit muscle protein kinase type II was tested as a possible substrate for the menadione-dependent phosphatase from *Clostridium sticklandii*. Labeled phosphate was released from the [^{32}P] Histone in the presence of the phosphatase. This observation, along with earlier results which showed that the menadione dependent phosphatase can hydrolyze [^{32}P] casein, suggest that peptide containing O-phosphoserine or O-phospho threonine might be the natural substrate of the phosphatase.

(2) Crude extracts of *C. sticklandii* which had been specifically freed of a menadione-dependent alkaline phosphatase by passage over a quinone affinity column were reacted with [γ^{32}P] ATP. When the ^{32}P-labeled extract protein was chromatographed and tested as substrate for the purified phosphatase, ^{32}P liberation was observed.

Proposed Course of Action

(1) The selenoprotein of the glycine reductase complex is considered a likely component of the system that might undergo phosphorylation and further attempts will be made to phosphorylate this protein either enzymically or chemically. An S-phosphocysteine residue on this protein is chemically reasonable and might serve as a substrate for the phosphatase.

(2) Peptides containing serine and or threonine residues will be ^{32}P labelled and tested in order to determine the degree of specificity for the hydrolyzable phosphate ester linkage. Other well-characterized phosphoproteins will also be tested.

(3) The bacterium *Clostridium sticklandii* will be grown in media containing ^{32}P inorganic phosphate and cell free extracts made and analyzed for phosphate incorporation.

Keyword Descriptors

Menadione, Quinone-dependent phosphatase, sulphydryl enzyme, Clostridium sticklandii.

Publications

Stadtman, E. R., Hohman, R. J., Davis, J. N., Wittenberger, M, Chock, P. B., and Rhee, S. G.: Subunit Interactions of Adenylylated Glutamine Synthetase. The Lipmann Symposium on Concepts of Chemical Recognition in Biology, 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00235-02-LB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Properties of 8-hydroxy-5-deazaflavin-dependent $NADP^+$ reductase from Methanococcus vannielii.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Shigeko Yamazaki	Staff Fellow	LB	NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism & Bioenergetict	LB	NHLBI
	Lin Tsai	Research Chemist	LB	NHLBI

COOPERATING UNITS (if any)

Frederic C. Jacobson and Christopher Walsh ....Stereochemical Studies
(Massachusetts Institute of Technology)

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Laboratory of Biochemistry

SECTION

Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.4	PROFESSIONAL: 1.2	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

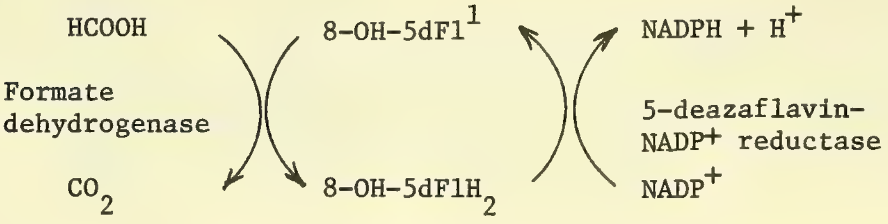
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

8-Hydroxy-5-deazaflavin-dependent $NADP^+$ reductase catalyzes a direct H-transfer reaction between 8-hydroxy-5-deazaflavin cofactor and nicotinamide dinucleotide phosphate. Stereochemistry of this reaction is determined as S-specific with respect to nicotinamide dinucleotide phosphate.

Project Description

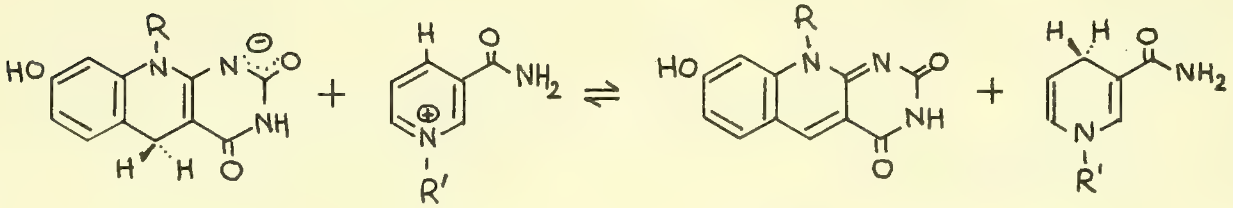
The formate-NADP$^+$ oxidoreductase system present in extracts of Methanococcus vannielii catalyzes the following overall reaction:



The aim of this project is to study the mechanism and stereochemistry of the reaction catalyzed by the 5-deazaflavin-NADP$^+$ reductase and to study properties of the 5-deazaflavin-NADP$^+$ reductase.

Major Findings

5-deazaflavin-NADP$^+$ reductase catalyzes the reaction:



8-OH-5dFlH$_2$ (I) NADP$^+$ 8-OH-5dFl (II) NADPH

For the forward reaction, the pH optimum is 7.9 with a 50% activity at pH 6.9 which is the pKa value of the N-1 position of 8-OH-5dFlH$_2$. The reverse reaction, however, shows a pH optimum at 4.8 and a 50% activity at pH 6.3, which is the pKa value of the 8-OH function of 8-OH-5dFl. These observations indicate that the N-1 anion (I) and the neutral molecule (II) are the reacting species for the forward and reverse reactions, respectively.

---

[1] The abbreviations used are: 8-OH-5dFl, 8-hydroxy-5-deazaflavin cofactor; 8-OH-5dFlH$_2$, 1,5-dihydro-8-hydroxy-5-deazaflavin cofactor; 5-deazaflavin-NADP$^+$ reductase, 8-hydroxy-5-deazaflavin-dependent NADP$^+$ reductase.

100

The evidence for a direct H-transfer process catalyzed by the enzyme is furnished by result of reacting (5R,S)-[5-^{3}H]8-OH-5dF1H$_2$ and NADP$^+$. At the completion of the reaction 49% of ^{3}H was found in NADPH and 48% in 8-OH-5dFl. The stereochemistry of the direct H-transfer process was studied as follows:

1) The purified [4-^{3}H]NADPH from the above experiment was oxidized either by a 4S or a 4R-specific enzyme. Results of these experiments suggested that the [4-^{3}H]NADPH enzyme product is (4S)-[4-^{3}H]NADPH.

2) The reduction of 8-OH-5dFl with either 4S or 4R-[4-^{3}H]NADPH in the presence of 5-deazaflavin-NADP$^+$ reductase showed that the transfer of ^{3}H occurred only between (4S)-[4-^{3}H]NADPH and 8-OH-5dFl producing [5-^{3}H]8-OH-5dF1H$_2$.

These results show that the stereochemistry of the direct H-transfer for both forward and reverse reactions is S-specific (or B-specific) with respect to nicotinamide dinucleotide phosphate.

5-Deazaflavin-NADP$^+$ reductase contains 2 gram atoms of Zn per mole, half of which is readily removed with a concomitant loss of 50% of enzymatic activity.

Proposed Course of Action

(1) Further studies of physical and chemical properties of 5-deazaflavin-NADP$^+$ reductase.

(2) The mechanism and the stereochemistry of the reaction catalyzed by the formate-NADP$^+$ oxidoreductase system will be studied.

Publications

Yamazaki, S. and Tsai, L.: Properties of 8-hydroxy-5-deazaflavin-dependent NADP$^+$ reductase from *Methanococcus vannielii*. Abs. 490 71st American Society of Biological Chemists Meeting, June 1-5, 1980.

Yamazaki, S. and Tsai, L.: Purification and properties of 8-hydroxy-5-deazaflavin-dependent NADP$^+$ reductase from *Methanococcus vannielii*. (1980) *J. Biol. Chem.* in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00236-02 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of Enzyme Degradation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael R. Maurizi	Staff Fellow	LB NHLBI
Other:	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.1	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Glutamate dehydrogenase (GDH) and lysine-sensitive aspartokinase (AKIII) are inactivated in Escherichia coli cells starved for ammonia or glucose. Inactivation of GDH and AKIII is blocked in relA mutants during ammonia starvation, but occurs normally in cya (cAMP-deficient) and deg (degradation-deficient) mutants.

Immunochemical assays indicate that both GDH and AKIII are degraded during inactivation. No fragments of either enzyme have been positively identified in immunoprecipitates obtained during degradation, but deg mutants do accumulate at least one lower molecular weight peptide that binds to an anti-GDH antibody column.

Purified GDH is highly resistant to proteolysis by proteases such as trypsin, chymotrypsin, subtilisin, and thermolysin. When NADPH is present at 10-200 μM, GDH is rapidly degraded by these same proteases. Proteolysis in the presence of NADPH can be prevented by α-ketoglutarate, glutamate, and numerous other anionic metabolites. GDH is stable in crude extracts of E. coli prepared by several methods, with or without NADPH. GDH in cells permeabilized with toluene undergoes a slight (10-30%) and variable loss of activity which is dependent on NADPH.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: In growing cells, proteins containing amino acid analogs (abnormal proteins), incomplete proteins (nonsense fragments), and free subunits of multimeric proteins (e.g., ribosomal subunits) are rapidly degraded, but most native proteins are stable. In starving cells, degradation of the various denatured proteins continues, and, in addition, many normal proteins begin to be degraded. The changes in the proteolytic system or in the native proteins that are responsible for the increased degradation of normal proteins in starving cells are not known.

The primary objectives of this project are: (1) to define the steps, in particular, the initial step, in the degradation of a native enzyme; (2) to determine the mechanism of regulation of the degradation of a native enzyme; (3) to identify the components of the degradative system responsible for degradation of native proteins.

Major Findings

Of the 18 enzymes examined in ammonia-starved *E. coli*, glutamate dehydrogenase (GDH) and lysine-sensitive aspartokinase (AKIII) were most rapidly inactivated. Inactivation of GDH (~ 35%/hour) and AKIII (~ 20%/hour) coincides with the increase in general protein turnover (~ 4%/hour) that occurs in ammonia-starved cells. That inactivation of GDH and AKIII is prevented when protein degradation is blocked (e.g., by chloramphenicol, azide, PMSF) suggests (but does not prove) that loss of activity of the enzymes results from degradation. Inactivation of GDH and AKIII does not occur during ammonia starvation in *relA* mutants in which protein turnover is also blocked. These results may indicate only that inactivation of these enzymes and increased degradation of cell protein are favored by the same physiological conditions.

Direct immunoprecipitation of GDH and AKIII from crude cell extracts using antibodies prepared against the purified enzymes demonstrated that both GDH and AKIII were degraded in cells during or following inactivation. The recovery of enzyme protein was determined after immunoprecipitation from extracts of cells that had been labeled with ^{3}H-leucine and chased with cold leucine during the starvation period. Following separation of the immunoprecipitates on SDS gels, recovery of radioactivity in GDH was consistently lower than recovery of activity, indicating that the GDH subunit is degraded (or drastically altered) very rapidly during inactivation. The lower recovery of radioactive protein mostly likely resulted from continued synthesis of the GDH during the chase with unlabeled leucine. Initial determinations of the recovery of radioactivity in AKIII indicated that inactive AKIII accumulated during inactivation; that is, degradation was slower than inactivation. Subsequent measurements have not produced the same result, and it is most likely that degradation proceeds rapidly upon inactivation. Whether there is an inactivation step prior to degradation of AKIII is still an open question (see Niles, E.G. and Westhead, E.W. (1973) Biochemistry 12, 1723-1729). Attempts to detect covalent modification of AKIII by measuring incorporation of ^{32}P (in any metabolic form) into AKIII showed less than .01 moles ^{32}P per mole of subunit in AKIII isolated from cells during ammonia starvation.

Proteolytic fragments of a protein would not necessarily be recovered in direct immunoprecipitates, and, in fact, immunoprecipitates of GDH or AKIII from cells contain only the intact subunits and no lower molecular weight peptides. When cell extracts were passed through affinity columns composed of anti-GDH or anti-AKIII antibodies covalently coupled to Sepharose 4B, several lower molecular weight peptides were isolated. The peptides appeared during the period of degradation and declined when the enzymes were nearly completely degraded. The amount of these peptides bound to anti-GDH antibody was higher in deg mutants which are deficient in the degradation of abnormal proteins, but which inactivate GDH at the same rate as wild type cells. The identity of these peptides as degradation products of GDH remains to be established.

Escherichia coli cells that have been washed and frozen retain the ability to inactivate GDH when shaken in buffer lacking an ammonia source. GDH is stable, however, in cell extracts prepared by a variety of techniques. Thus, rupturing cells causes the inactivation, inhibition, or excessive dilution of components necessary for GDH inactivation and degradation. Attempts to inactivate GDH in cells permeabilized with toluene have given mixed results. Incubation of permeabilized cells at 30°C in the presence of NADPH results in the loss of 10-30% of the GDH in 30 minutes. The control, with no NADPH present, shows no loss of GDH. This result is highly variable, however, and degradation of GDH under these conditions has not been shown. The lability of AKIII, which is generally more labile in cell extracts and permeabilized cells, has not been studied in vitro in any detail.

The properties of purified GDH in the presence of substrates and ligands may provide a model of how degradation of GDH is regulated. Purified GDH in the absence of ligands is highly resistant to proteolysis when tested with trypsin, chymotrypsin, subtilisin, thermolysin, or pronase. Low concentrations of NADPH (10-200 μM) cause GDH to become highly susceptible to degradation, and GDH can be protected from this effect by α-ketoglutarate, glutamate, and numerous other anionic compounds. Only NADPH has been found to destabilize GDH; no other compounds tested -- most notably NADP, NAD, NADH, or nucleotides -- has the effect on the enzyme.

The mechanism by which NADPH destabilizes GDH is not known. NADPH is not consumed during the incubation. At high concentrations of GDH, NADPH does not cause a change in light scattering and therefore probably does not cause association or dissociation of the enzyme. An NADPH induced conformational change is suggested by a perturbation of the ultraviolet spectrum of the enzyme and by the exposure of one sulfhydryl group to rapid reaction with dithiobis-(nitrobenzoic) acid. At low concentrations of GDH, NADPH results in the rapid, irreversible loss of GDH activity. Many anionic metabolites prevent NADPH-dependent inactivation of GDH, but much less protection is obtained with amino acids and other amines.

GDH is not inactivated by ascorbate under conditions in which E. coli glutamine synthetase is rapidly inactivated. Treatment of GDH with ascorbate, NADH, phenazine methosulfate, or ferrous iron does not increase susceptibility of GDH to proteolysis by trypsin or subtilisin. High concentrations (100 mM) of H_2O_2 inactivate GDH, alter its migration in SDS gels, and make it highly susceptible to proteolysis. Although GDH is not highly sensitive to oxidative or reductive inactivation, such a step in the inactivation of the enzyme *in vivo* cannot be ruled out at this time.

Relevance to Biomedical Research

Metabolic regulation is accomplished in part by control of enzyme levels which in turn are determined by the sum of synthesis and degradation of the enzymes. Clearly, the degradation of enzymes must be regulated, since uncontrolled degradation would be harmful and wasteful. It is known that alterations in the degradative systems are part of the physiological consequences of starvation and aging, and of such diseases as diabetes and muscular dystrophy. In addition, selective degradation of proteins may be important during viral infections and during cellular defferentiation.

In most cases, the degradative systems of cells are incompletely understood. Very little is known concerning the initiation and control of degradation of individual enzymes. Since protein turnover in *E. coli* shares at least two important features with turnover in mammalian cells, *i.e.*, selectivity and energy dependence, *E. coli* is a convenient model system in which to study this process.

Proposed Course of Action

1. Attempts will be made to isolate larger quantities of the lower molecular weight proteins that bind to the anti-GDH antibody affinity column. If larger quantities can be obtained, procedures such as end-group analysis and peptide mapping will be used to establish their identity of degradation products of GDH.

Mutants lacking one or several peptidases will also be examined to see if degradation products of GDH or AKIII accumulate during inactivation.

2. Several *in vitro* systems for the degradation of proteins have been described in the literature recently. One or more of these systems can be tested for its ability to inactivate and degrade GDH or AKIII. Degradation alone can be tested by using GDH inactivated with NADPH.

3. Efforts to obtain a permeabilized cell system capable of degrading GDH or AKIII will be continued.

4. Further studies of the effect of NADPH on GDH will be conducted. Ultracentrifugation will give a more sensitive measure of molecular weight changes. Additional tests can be made if the altered exposure of reactive residues *e.g.*, lysines, arginines, histidines, tryptophans, or others. The individual steps in the degradation will be analyzed to determine if one specific site is exposed upon NADPH binding.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00237-01 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Toxicity and Transport of Bilirubin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Rodney L. Levine	Senior Surgeon, U.S.P.H.S.	LB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.125	0.1	0.025

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hyperbilirubinemia is probably the most frequent disease which is diagnosed and treated in the human newborn. Current treatment attempts to prevent neurologic damage. Treatment is based on the theory that free (unbound) bilirubin is the toxic fraction of the total bilirubin pool. Proof of this theory is lacking, and certain clinical and experimental findings are not consistent with the theory. Another hypothesis better explains the available data: bilirubin enters the brain bound to albumin when the blood-brain barrier opens. This hypothesis is amenable to experimental testing.

Project Description

Introduction

Bilirubin is an end-product of heme metabolism in mammals. In vitro, bilirubin is very toxic to cells and to cellular components such as mitochondria. The biochemical basis of the toxicity has not been established.

Hyperbilirubinemia is probably the most commonly diagnosed and treated medical problem of newborn humans. The purpose of treatment is to avoid transport of bilirubin into the brain. If transport and deposition occur, they lead to devastating neurologic damage and the syndrome of kernicterus.

Major Findings

A critical review of neonatal jaundice was undertaken. I found that virtually all medical thinking is based on the premise that free bilirubin (i.e., non-albumin bound) is the toxic fraction of the total pool. Yet no evidence is available to establish this as fact. Further, virtually nothing is known about the mechanism of transport of bilirubin across the blood-brain barrier.

Many experimental and clinical findings appear inconsistent with the free bilirubin theory of transport and toxicity. A new hypothesis was proposed: Under certain conditions, the blood-brain barrier opens -- sometimes reversibly. If this occurs in the presence of hyperbilirubinemia, then albumin-bound bilirubin will gain access to the brain substance. This hypothesis can explain virtually all of the clinical and experimental characteristics of kernicterus.

Significance to Biomedical Research

Current treatment of neonatal hyperbilirubinemia is based on the free bilirubin theory, an unproven concept. If this is not the mechanism of transport and toxicity, then treatment programs will require detailed reassessment.

Proposed Course of Research

The major goal will be to test the hypothesis that kernicterus results when the blood-brain barrier opens in the presence of hyperbilirubinemia. This work will be done in collaboration with Dr. Stanley Rapoport of the National Institute on Aging.

Publications

Levine, R. L: Bilirubin: Worked out years ago? Pediatrics 64: 380-385, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00238-01 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Selenium Proteins from Clostridium kluyveri

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Maris G. N. Hartmanis	Visiting Investigator (Swedish support) (terminated 7/18/80)	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.0	OTHER: 0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

When grown in media containing 1 μM selenite Clostridium kluyveri specifically incorporates selenium into the enzyme thiolase. This has been shown by using protein separation techniques such as gel filtration and gel electrophoresis. Thiolase in Clostridium kluyveri exists in two forms one of which contains selenium. The selenium-containing thiolase is more active than the non-selenium thiolase.

PHS-6040
(Rev. 10-76)

Project Description

Isolation and characterization of a selenium-containing thiolase from *Clostridium kluyveri*.

Major Findings

The enzyme thiolase from *Clostridium kluyveri* has been shown to exist in two forms both of which have been purified to near homogeneity. The most active of these forms has been shown to be a selenium-containing protein by the following criteria: coelution of selenium with thiolase activity after gel filtration, comigration of protein and ^{75}Se in SDS-disc gel electrophoresis, comigration of ^{75}Se and protein showing thiolase activity using gel electrophoresis under non-denaturing conditions. The tentative identification of thiolase as a selenium-containing enzyme provides the first example of the occurrence of selenium in a non-redox catalyst.

The selenium-containing component has not yet been characterized.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00239-01 LB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation and Mechanism of Glutamine Synthetase in E. coli

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sue Goo Rhee	Research Chemist	LB NHLBI
	Emilio Garcia	Staff Fellow	LB NHLBI
Others:	P. B. Chock	Research Chemist	LB NHLBI
	R. J. Hohman	Chemist	LB NHLBI
	E. R. Stadtman	Chief, Laboratory of Biochemistry	LB NHLBI
	M. D. Stamatakos	Summer Student (6/4-8/30/80)	LB NHLBI

COOPERATING UNITS (if any)
Gregory Ubom, Chemistry Department, The Catholic University of America, Washington, D. C.

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Enzymes

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2.5	PROFESSIONAL: 2.3	OTHER: 0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

(1) The bifunctional enzyme responsible for the uridylylation and deuridylylation of P_{II} protein in Escherichia coli was purified to homogeneity. The molecular weight was determined under native and denaturing conditions and found to be 110,000 ~ 100,000, indicating that this protein consists of a single polypeptide. (2) The mechanism of adenylylated glutamine synthetase was studied using the glutamine synthetase adenylylated with fluorescent and spin labeled analogues of ATP, 2-aza-1,N-etheno-adenosine triphosphate and 2,2,6,6,-tetramethyl piperdine-1-oxyl (Tempo) ATP. The ligand induced fluorescent changes were used to evaluate the thermodynamic and dynamic properties of the complexes formed between the adenylylated enzyme and substrates. Analysis of the stopped-flow kinetic data indicates that the biosynthetic reaction catalyzed by the Mn-dependent adenylylated glutamine synthetase proceeds through a random binding of substrates followed by sequential reactions involving at least four fluorometrically distinctive intermediates. Distances between the nitroxide group at the adenylylation site and Mn at n_1 (the structural binding site) and n_2 (the nucleotide binding site) were evaluated in the presence and absence of substrates.

Project Description

Objectives: (1) The cascade control of glutamine synthetase (GS) in *Escherichia coli* involves two types of reversible covalent modifications, namely, adenylylation of GS and uridylylation of P_{II}. Although the adenylylation-deadenylylation cycle has been studied in detail, studies on the complete bicyclic system was not possible due to the unavailability of the enzyme responsible for the uridylylation (UT enzyme) and deuridylylation (UR enzyme). The present work involves the purification and characterization of UT and UR enzyme(s). (2) Previously, the thermodynamic and kinetic properties of unadenylylated GS have been characterized. In an effort to further understand the mechanism of action of GS, we are studying the properties of the adenylylated enzyme.

Major Findings

(1) Purification of Uridylyltransferase and Uridylyl-Removing Enzyme from E. coli (with E. Garcia). This project was undertaken in collaboration with Dr. Emilio Garcia. For details on the purification and characterization of UT-UR enzyme, see Dr. Garcia's report. In addition, a simplified procedure for the purification of P_{II} protein has been established. Since the P_{II} proteins are substrates for the UR-UT activities, and are present in extremely low concentrations in *E. coli*, an efficient method for the isolation of P_{II} is essential for future studies on the UR-UT enzyme. It is also hoped that storage of UT-UR in the presence of P_{II} will improve its stability.

(2) Mechanism of Adenylylated Glutamine Synthetase (with G. Ubom and P. B. Chock). Prior to this study, the catalytic cycle of the biosynthetic reaction catalyzed by the Mg(II)-dependent unadenylylated GS had been elucidated by monitoring the intrinsic protein fluorescence change accompanying substrate binding and the catalytic reaction. However, due to a negligible fluorescence change, a similar study could not be done with the adenylylated enzyme. In this study, the AMP moiety of the adenylylated GS was replaced by a fluorescent analog of AMP, 2-aza-1, ^{6}N-etheno AMP (aza-AMP), and by a spin labeled AMP derivative, 2,2,6,6-tetramethyl piperidine-1-oxyl (Tempo) AMP, making it possible to study this enzyme using fluorometric and epr techniques.

a. Fluorometric studies on 2-aza-1, ^{6}N-etheno adenylylated glutamine synthetase (aza-GS).

(a-1) Characterization of the aza-GS. The number of aza-AMP attached to GS determined by the γ-glutamyltransferase assay and UV spectrum was 9 ~ 10 per dodecamer. Two empirical formulas were deduced to relate the enzyme concentration, X (mg/ml), and the number of aza-AMP attached per dodecamer, n, with absorbances at 340, 290 and 240 nm.

$$A_{240} = 1.50\ X + 0.0625\ nX + 4.16\ (A_{340} - 0.0033\ nX)$$

$$A_{290} = 0.385\ X + 0.00917\ nX + 1.89\ (A_{340} - 0.0033\ nX)$$

Unlike the unmodified AMP, absorption maximum of aza-AMP is 240 nm, and absorption at 290 and 340 nm is significant.

As shown in Table 1, the aza-GS exhibits catalytic properties and kinetic properties similar to those of the naturally adenylylated GS.

Table 1. Comparison of the Biosynthetic Reaction Catalyzed by Aza-GS and $\mathrm{GS}_{\overline{12}}$

		$\mathrm{GS}_{\overline{12}}$	Aza-GS
Specific activity at 10°C		0.553 unit	0.684
Optimal pH		6.7	6.3
Mn supported activity		+	+
Mg supported activity		-	-
Km	ATP	5×10^{-5} M	3.3×10^{-5} M
	Glu	6×10^{-3} M	5×10^{-3} M
	NH_3	1×10^{-4} M	1.3×10^{-4} M

(a-2) Thermodynamic properties. The fluorescence spectrum of aza-GS exhibits two excitation maxima at 300 and 368 nm, and an emission maximum at 470 nm. The emission quantum yield is dependent on the excitation maximum used and is sensitive to the conformational changes induced by the binding of divalent metal ions and substrates, and by the formation of reaction intermediates. These changes in fluorescence intensity were used to monitor the binding of substrates to the Mn(II) activated aza-GS for both the transferase reaction (ADP, L-gln, ASO_4) and the biosynthetic reaction (ATP, L-glu). Binding of NH_3 to the aza-GS does not cause any significant signal changes unless ATP and glu are present. All data in Table 2 were obtained at pH 7.2, 20°C, 50 mM Hepes buffer containing 100 mM KCl and 1 mM Mn.

Table 2. Dissociation of the Biosynthetic and γ-Glutamyl Transfer Substrates

Reaction	Kdiss , M
EMn + ATP	5×10^{-5}
EMn·L-glu + ATP	3.5×10^{-5}
EMn·ATP + L-glu	4×10^{-3}
EMn + ADP	1.6×10^{-4}
EMn·L-gln + ADP	5.2×10^{-4}
EMn + L-gln	1.7×10^{-3}
EMn·ADP + L-gln	8.9×10^{-3}
EMn·ASO_4 + ADP	1.8×10^{-5}
EMn·ADP·L-gln + ASO_4	4×10^{-4}
EMn·ASO_4 + L-gln	10×10^{-3}
EMn·ASO_4·L-gln + ADP	3×10^{-6}
EMn·ADP + ASO_4	2.4×10^{-4}

The results in Table 2 show that all substrates bind randomly. Unlike Mg(II) activated unadenylylated enzyme, there is no synergistic interaction between L-glu and ATP. In the γ-glutamyl transfer system, L-gln antagonizes the binding of ADP and ASO_4, while ADP and ASO_4 enhance each other's affinities.

In contrast to the Mn(II) aza-GS, the catalytically inactive Mg(II) aza-GS produced only a small fluorescence change upon the binding of ATP and L-glu, and no change upon the subsequent addition of NH_3 to the Mg·aza-GS·ATP·L-glu complex. This observation provides direct evidence of conformational differences for the Mn(II) and Mg(II) forms of the adenylylated enzyme. The binding of two glutamate analogs, L-methionine-S-sulfoximine and L-methionine-R-sulfoximine to Mn(II) aza-GS was also examined. Results indicate that both diastereomers bind with negative cooperativity; the S-isomer with $S_{1/2} = 63$ μM, $n_H = 0.70$ and R-isomer with $S_{1/2} = 420$ μM, $n_H = 0.87$.

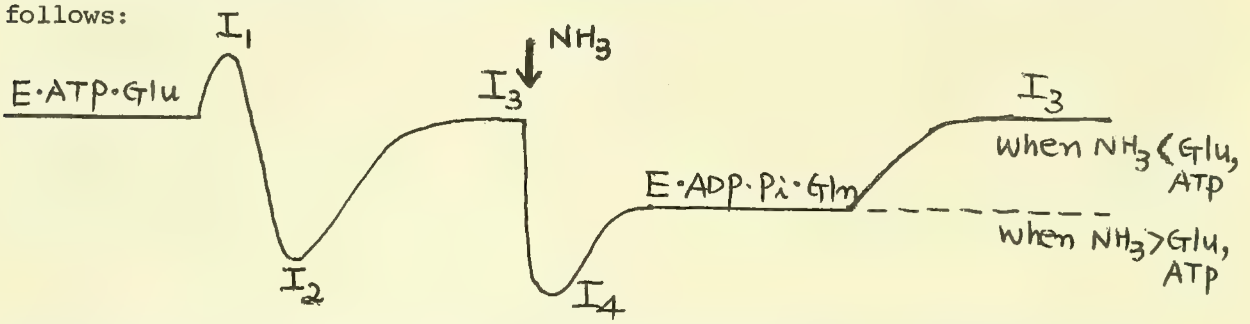
(a-3) Rapid kinetics. The time course of the fluorescence change for the tightening of the divalent metal-free aza-GS was monitored. The results indicate that the reaction proceeds with a very rapid step followed by a relatively slow step ($t_{1/2} = 2.0$ minutes at 20°C) for both Mg(II) and Mn(II).

The fluorescence changes induced by substrate binding and intermediate formation were followed by stopped-flow analysis. Nineteen mixing experiments listed below are possible for the biosynthetic reaction system which involves three substrates (T = ATP, G = L-glu, N = NH_3).

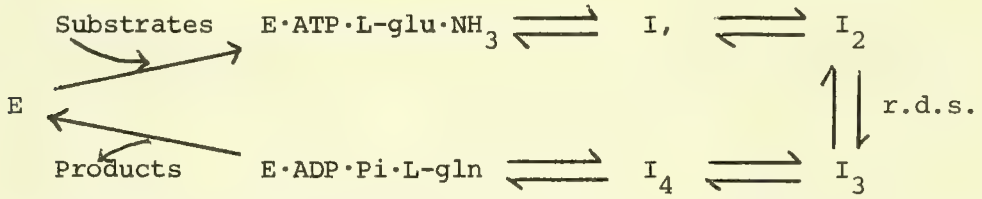
(1) E + G
(2) E + T
(3) E + N
(4) ET + G
(5) EG + N
(6) EG + T
(7) ET + N
(8) EN + G
(9) EN + T
(10) E + GT
(11) E + TN
(12) E + GN
(13) EN + GT
(14) ET + GN
(15) EG + TN
(16) EGT + N
(17) EGN + T
(18) ETN + G
(19) E + GTN

As previously mentioned NH_3 (N) causes neither fluorescence changes, nor produces any significant effect on the binding of the other substrates, unless all three substrates are present. Therefore, reactions 3, 5, 7, 8, 9, 11, and 12 were not studied in detail. All reactions were performed at 10°C in 50 mM Hepes buffer, pH 7.2, containing 0.1 M KCl and 1.0 mM Mn. The mechanism of binary complex formation from E and T, and E and G could not be studied in detail because of their rapid fluoresence changes. The rate of reaction 1 measured at the excitation wave length of 300 and 368 nm was very rapid ($t_{1/2} = 2$ msec when G = 1.5 mM) and increased with increasing concentration of G. The signal change of reaction 2 was very rapid ($t_{1/2} < 2$ msec) and its amplitude was small. For reactions 4, 6, and 10, addition of Glu and ATP causes, regardless of the order of their addition, a triphasic time-dependent exponential change of fluorescence when excited at 300 nm. The first rapid increased fluorescence change with an apparent half-life of 30 msec was followed by a decrease (apparent $t_{1/2} = 400 \sim 600$ msec), and then by an increase of an apparent $t_{1/2} = 1.5$ sec. When excited at 368 nm, a biphasic change involving a decrease with an apparent $t_{1/2} = 400$-600 msec and an increase of a small amplitude with an apparent $t_{1/2} = 1.5$ sec was observed. Addition of NH_3 into EGT (reaction 16) caused a rapid concentration-dependent decrease of fluorescence ($t_{1/2} = 32$ msec when N = 0.25 mM, $t_{1/2} = 4$ msec when N = 40 mM), followed by an increase with $t_{1/2} = 150$ msec. Then a steady-state phase followed.

If NH_3 is the limiting substrate, the signal rises slowly again after a lag time. The lag time increased proportionally when NH_3 concentration was increased, or when enzyme concentration was decreased, suggesting that during the lag time steady-state consumption of NH_3 was occurring. The schematic representation of the fluorescence change during a cycle of catalysis is as follows:



The results from this rapid kinetic study indicate that the biosynthetic reaction catalyzed by the Mn(II)-dependent adenylylated enzyme proceeds through a quaternary complex formed from a random binding of ATP, L-glu, and NH_3, followed by four fluorometrically distinctive intermediates (I_n).



The results obtained from other reactions (reactions 13-18) also support this scheme. The rate determining step in this cycle is the conversion of I_2 to I_3, which later reacts with NH_3 to give I_4.

Current studies do not lead to any conclusion of the chemical nature of the enzyme bound intermediates formed during the catalytic cycle. However, various results obtained from chemical and isotope exchange studies suggested that γ-glutamyl phosphate and ADP are formed from ATP and L-glu before NH_3 addition. Therefore, it is conceivable that two fluorometrically sensitive conformational changes (I_1 and I_2) occur before γ-glutamyl phosphate and ADP are formed in the rate determining step. Then NH_3 reacts with the γ-glutamyl phosphate to produce L-gln and Pi (I_4). Finally, another conformational change takes place before products are released.

(b). EPR studies 2,2,6,6-tetramethyl piperidine-1-oxyl (Tempo) adenylylated glutamine synthetase (Tempo-GS). The number of tempo-AMP attached covalently to GS was 7.5-8.5 when measured by (i) the γ-glutamyl-transfer assay, (ii) the UV spectrum, and (iii) the AMP fractional inhibition. The pH profile of the Mn(II)-supported biosynthetic activity was similar to that of naturally adenylylated GS.

The correlation time for tempo-GS is 2.1×10^{-10} sec compared to 8.1×10^{-10} sec for tempo-ATP. This indicates that the tempo moiety of the adenylylated GS is only slightly immobilized. When Mn(II) was titrated into the tempo-GS, there was a progressive decrease in the amplitude of the epr spectrum due to the spin-spin interaction between the nitroxyl radical at the adenylylation site and Mn(II) bound at n_1 and n_2 sites. The dissociation constant of Mn(II) to n_1 and n_2 was 1.8 μM and 18 μM when measured by equilibrium dialysis. The extent of the signal decrease was related to two separate distances γ_1 and γ_2, which represent the distances between the bound Mn(II) at n_1 and n_2 sites and the nitroxide spin. The distances γ_1 and γ_2 are 20 Å and 18 Å, respectively.

When the same type of titration was performed in the presence of ADP and Pi, ATP and L-glu, and ADP and L-gln, significantly different titration curves were generated. Qualitative analysis shows that in the presence of various substrates γ_1 becomes shorter, while γ_2 remains constant. Quantitative analysis requires determination of two dissociation constants for the Mn(II) binding to n_1 and n_2 sites in the presence of the substrates. It is known that the presence of effectors and substrates affect only the binding of Mn(II) at the catalytic site (n_2), but not at the structural site (n_1). The Mn(II) dissociation constant at n_2 site was found to be 3 μM for the L-gln-ADP system, 10 μM for the L-glu-ATP system, and 5 μM for the ADP-Pi system. Using these data and the epr titration curves, quantiative evaluation of γ_1 and γ_2 is in progess.

(3) Adenylylation of Glutamine Synthetase with a Photo-Affinity ATP Analog and Preparation of Antibodies Specific for Aza-AMP (with R. Hohman and E.R. Stadtman. Realizing that fully adenylylated GS contains 12 precisely defined antigenic determinants for AMP specific antibodies and that the epitope density varies as the state of adenylylation changes, Hohman and Stadtman investigated the interaction between GS at various states of adenylylation and AMP specific antibodies prepared by immunizing a sheep with an AMP-BSA conjugate. Their results indicate that the multideterminant antigen-antibody interaction is not simple. Characterization of this interaction might be achieved by using a sensitive reporting group on the antigen-antibody complex or by linking the antigen-antibody complex covalently. The aza-GS is an ideal antigen with a sensitive reporting group, because the aza-AMP attached to the active center of the antigen-antibody interaction shows a sensitive fluorescence change, and because its excitation maximum at 368 nm eliminates the filter effect of protein. Therefore, the sheep antibodies against the aza AMP-BSA conjugate have already been prepared. As a way to attach an antibody covalently to the antigenic site, the azido-GS was prepared by adenylylating GS with a photo-affinity ATP analog, 8-azido ATP (prepared by Dr. B. Haley, University of Wyoming). Further characterization of this azido-GS is in progress.

Proposed Course of Action

1. With the purified UT-UR enzyme, allosteric effects of various effectors, particularly α-ketoglutarate, L-gln, ATP, Mg(II) and Mn(II) will be studied. The complete bicyclic system will then be investigated.

2. Antibodies specific for P_{IIA}, P_{IID}, and UT-UR will be prepared for future genetic and in vivo studies.

3. Formation of a covalently linked adduct between the azido-GS and the

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azido-AMP specific antibodies will be followed at various protein concentrations and time intervals. The adducts will then be separated by size before characterization with the electron microscope.

4. Previous kinetic studies have suggested that the adenylyltransferase is a bifunctional enzyme with adenylylation and deadenylylation activities. Direct support for this thesis will be sought by blocking the deadenylylation active center with azido-GS and by demonstrating that the azido-GS·ATase adduct still can catalyze the adenylylation.

5. Using a rapid kinetic and light scattering technique, kinetic studies on the interaction of aza-GS and aza-AMP specific antibodies will be pursued.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00240-01 LB

PERIOD COVERED
January 27, 1980 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Purification and characterization of selenium-containing transfer RNAs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Wei-Mei Ching	Staff Fellow	LB NHLBI
Other:	Thressa C. Stadtman	Chief, Section on Intermediary Metabolism and Bioenergetics	LB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemistry

SECTION
Section on Intermediary Metabolism and Bioenergetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.6	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Reversed phase chromatography (RPC-5) and reversed salt gradient chromatography (Sepharose 4B) have been set up recently. A ^{75}Se-tRNA, separated from Pseudomonas cells, was identified as L-leucyl-tRNA and further separated from its non-selenium containing isoacceptors by RPC-5. A quite pure seleno-tRNA from C. sticklandii was separated from its bulk tRNA mixture after two reversed salt gradient chromatographic steps and two reversed phase chromatographic steps. Preliminary results indicate this seleno-tRNA exhibits an unusual absorbance at 291 nm which may be a contribution of the seleno base present in the preparation.

Purpose:

Selenium is found to be incorporated into tRNAs of Clostridium sticklandii, Methanococcus vannielii and a few other anaerobic bacteria. This incorporation process is highly specific and can not be diminished by excess sulfur. Up to date, the biological significance of selenium-containing tRNA is unknown. In order to pursue the nature of these seleno-tRNAs, it is necessary to develop separation methods for purifying seleno-tRNAs to homogeneity.

Proposed Action

(1) Isolation of amino acid-tRNA synthetase from the same organism as for seleno-tRNA separation. This is necessary for the identification of tRNAs. Isolation from C. sticklandii is in process.

(2) Purification of seleno-tRNA by the conjunction of RPC-5, Sepharose 4B and DEAE Sephadex A-50.

(3) Characterization of the purified seleno-tRNA

- (a) Identify the cognate amino acid.
- (b) Determine the number of selenium atoms, or number of seleno-bases per seleno-tRNA.
- (c) Characterize and compare the suspected selenium containing nucleosides (isolated by HPLC after enzymatic digestion) from different seleno-tRNAs.
- (d) Study the effect of de-selenization of seleno-tRNA on charging activity.
- (e) Study the codon-anticodon recognition by ribosome binding activity.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00241-01 LB

PERIOD COVERED

January 1, 1980 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Detection of proteins in procaryotes and eucaryotes containing tyrosine residues modified by phosphate or nucleotide phosphate.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Todd Martensen	Staff Fellow	LB NHLBI
Other:	None		

COOPERATING UNITS (if any)

none

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Section on Enzymes

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.6	0.5	0.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

E. coli contains two proteins which are covalently linked to nucleoside monophosphates (AMP and UMP) through a tyrosine phosphodiester bond. Recently, it has been shown that mammalian cells contain phosphorylated tyrosine residues in proteins, and in one case a viral protein covalently linked to polynucleotide through a tyrosyl phosphodiester bond. Present work involves the detection and quantitation of proteins containing either of these modifications by special protein hydrolysis techniques for tyrosine phosphate and specific antibody recognition for adenylylated and uridylylated tyrosine.

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Introduction

E. coli contains two proteins which are covalently modified by the esterification of a tyrosyl residue to the 5'-phosphoryl moiety of nucleoside monophosphate: Glutamine synthetase (AMP) and P_{II} (UMP). Recently, it has been shown that mammalian proteins of cells transformed by oncogenic virii contains phosphorylated tyrosine. A nontransforming related virus (polyoma) has been shown to cause mammalian cell cultures to produce a viral protein covalently linked to polynucleotide through a tyrosyl phosphodiester bond. No technique exists to date to quantitate these amino acid modifications. Thus, a survey of cells or tissues for these modified amino acids awaits a simple technique. Glutamine synthetase is a model protein to study the quantitation of both amino acid modifications.

Major Findings

1) Stability of phosphorylated tyrosine was studied under conditions used for hydrolysis of proteins. It was found that acidic hydrolysis conditions were unfavorable for the quantitation of tyrosine phosphate but could be used to prepare peptides containing phosphotyrosine and sufficient free phosphotyrosine for qualitative detection.

2) Phosphorylated tyrosine was found to be stable during basic protein hydrolysis conditions. This technique should allow a straightforward methodology for the detection and quantitation of tyrosine phosphate in proteins with the amino acid analyzer.

3) Glutamine synthetase containing adenylylated tyrosine was treated with a specific nuclease to yield the enzyme containing phosphotyrosine. This is being studied as a model protein containing phosphotyrosine to determine the lability of the phosphotyrosine moiety in a protein and optimization of techniques for quantitation.

4) Synthesis of nucleotide-amino acid analogs. Synthesis of tyrosine covalently linked through the phenolic hydroxl to the 5' phosphoryl group of AMP was tried by three techniques. None of them appeared to yield the desired product. Synthesis of nucleotide phosphoryl derivatives of lysine to act as inhibitors to antibodies against nucleotide lysine adducts appears promising. The n-butylamine-5'-phosphoramidate of AMP was made to act as a competitive inhibitor of antibodies directed against the lysine epsilon amino phosphoramidate derivative of AMP. The efficacy of this compound is being tested.

5) Specificity of the P_{II} deadenylylation reaction was studied with a sample of ^{3}H-AMP-GS which was metal free and contained blocked sulfhydryl groups. This monomeric sample was not a substrate for the P_{II} catalyzed deadenylylation system demonstrating that tertiary and quaternary structural requirements are critical for GS to be recognized as a substrate.

Significance to Biomed Research

The association of phosphotyrosine and nucleotidylylated proteins with viral infiltration of cells is poorly understood. The development of specific assay systems based on using *E. coli* proteins as model systems should be very useful to elucidate the role of the modifications.

Proposed Course of Research

The major immediate goal is to develop specific assay systems for phosphotyrosine and nucleotidylyltyrosine. Then, a survey of bacterial and eucaryotic cells will begin.

Publications

None.

Annual Report of the
Cardiology Branch
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

The basic areas of experimental interest of the Cardiology Branch relate to the pathogenesis, pathophysiology and treatment of coronary artery disease and of hypertrophic cardiomyopathy; to the development of non-invasive techniques to assess cardiac structure and function; and to the elucidation of molecular mechanisms responsible for the contractile function of the heart. During the past year a major new direction of research has been launched: namely, defining 1) the role of calcium antagonist drugs in the treatment of coronary artery disease and hypertrophic cardiomyopathy, and 2) the mechanisms of action responsible for the beneficial effects of these drugs.

CALCIUM ANTAGONIST DRUGS

Calcium antagonists in the treatment of hypertrophic cardiomyopathy (HCM)

Although propranolol is effective in treating patients with HCM, many are unresponsive. The only alternative therapy in patients with obstruction is operation, which carries a 5-10% risk. No alternative is available to those without obstruction. We hypothesized that myocardial intracellular calcium overload may be a possible etiologic mechanism responsible for the hemodynamic abnormalities of patients with HCM. Last year we demonstrated that verapamil decreased LV outflow tract obstruction, improved exercise capacity, and favorably altered the symptomatic status in 19 patients with HCM. This year, to assess the chronic effects of verapamil in HCM, 78 patients whose lifestyle was unacceptable despite propranolol therapy were begun in hospital on oral verapamil. Forty-two patients have remained on verapamil for 6 to 30 months (median 14 months). Long-term verapamil led to improved treadmill exercise duration compared to in-hospital a) control (+ 53%; $p<.001$), b) propranolol (+ 32%; $p<.01$), c) in-hospital verapamil (+ 18%; $p<.05$). A total of 46% have either improved their functional class by at least one grade, have increased their exercise duration by 15%, or both.

However, potentially serious complications can occur as a result of verapamil's electrophysiologic and hemodynamic actions. In our series of 120 patients started on oral verapamil, these complications have included: 1) suppression of sinus node automaticity, manifested by sinus bradycardia (6%) or rarely as sinus arrest (2%); 2) inhibition of A-V nodal conduction, occasionally leading to second degree heart block (5%); 3) vasodilatation, which may lead to symptomatic hypotension (4%). Moreover, the dynamic nature of the obstruction to LV outflow in patients with obstructive HCM could potentiate the deleterious effects of the above complications. Thus, any complication leading to hypotension could augment LV obstruction. The resulting high LV pressure relative to the low coronary perfusion pressure would predispose to LV electrical or mechanical instability (8% of patients developed pulmonary edema).

Thus, we have now established verapamil as an important drug in the symptomatic treatment of HCM patients. However, potentially serious complications can occur and the drug must be used with caution.

To determine the mechanisms responsible for verapamil's beneficial actions, we examined the effects of oral verapamil on left ventricular (LV) systolic and diastolic function in patients with HCM. High resolution time activity curves derived from gated Tc99m radionuclide angiograms were analyzed before and after verapamil therapy in 50 patients, of whom 16 were also studied during propranolol therapy. Systolic function was normal or supranormal in all patients. Diastolic dysfunction was evident in 70% of patients, manifest by a decrease in peak LV filling rate (FR), and increased time to peak FR. While verapamil did not change LV systolic function, it substantially improved abnormalities in LV filling. In contrast, propranolol depressed LV systolic function and did not alter abnormalities in LV filling. Thus, verapamil may improve exercise capacity by diminishing LV outflow tract gradient, and by improving LV filling abnormalities common to patients with HCM.

A highly sensitive, reproducible high pressure liquid chromatography assay was developed, in collaboration with Dr. Henry Fales' laboratory, to assess the potential importance of plasma verapamil levels (PVL) in guiding therapy of patients with HCM. The relation of PVL to exercise capacity and functional status of 70 patients with HCM was studied. We found marked (up to 100%) interpatient variability in PVL at each dosage studied and no differences in PVL between clinical responders, nonresponders, and patients with serious side effects related to verapamil. Thus, PVL are of limited usefulness in predicting therapeutic or toxic effects in pts with HCM.

Calcium antagonists in the treatment of coronary artery disease (CAD)

Exercise performance: Propranolol has been one of the most effective drugs in treating patients with CAD. However, many patients are inadequately responsive to it and others suffer from significant side effects. Clinical studies from Europe suggest verapamil improves symptoms in patients with CAD, and recent studies in this country indicate an important therapeutic role of calcium antagonists in treating patients with coronary spasm. However, because no definitive studies are available concerning the efficacy of calcium antagonists in patients with stable CAD without evidence of spasm, we determined the effectiveness of oral verapamil alone and with propranolol in 11 such patients (9 refractory to beta blockers). Compared to placebo, verapamil (480 mg/day) improved duration of bicycle exercise in all patients (from 370 to 508 secs; $p<.001$) and was more effective than 160 to 320 mg/day propranolol (385 secs; $p<.005$ vs verapamil). Verapamil plus propranolol further increased exercise time (to 590 sec; $p<.01$ vs verapamil) and 9/11 pts (82%) were pain-free during exercise testing (in contrast to 2/11 with propranolol and 1/11 with verapamil). Time to 1 mm ST-segment depression was increased by both verapamil and verapamil plus propranolol. Thus, 1) verapamil was a more effective anti-anginal agent than propranolol and 2) verapamil plus propranolol provided additional improvement in exercise capacity over either drug alone.

Mechanisms of action: While verapamil decreases HR modestly during exercise, it does not consistently reduce pressure-rate product. Hence, the mechanisms responsible for its beneficial effects on exercise performance in patients with stable CAD are unclear. However, we found that 90% of all patients with CAD manifest abnormalities in LV filling (vida infra). Of note, verapamil improved both peak filling rate (PFR) and time to PFR in 9 of the 11 patients, in contrast to a lack of change following propranolol. Thus, enhanced LV

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diastolic filling may account in part for the symptomatic improvement observed in many CAD patients during verapamil therapy.

Negative inotropy: Although in vitro studies have demonstrated calcium antagonists have negative inotropic effects, controversy exists regarding the magnitude of such actions in vivo (and therefore the potential of these drugs to cause cardiac failure). We found verapamil decreased rest ejection fraction (EF) from 48 to 44% in patients with CAD ($p<.02$), but did not change ejection rate (ER). Rest EF decreased >5% in 5 of 11 patients (45%) on verapamil, and in 7/11 patients (64%) on verapamil plus propranolol. However, no patient developed clinical CHF and the drugs did not change exercise EF. Thus, in patients with reasonable baseline LV function (EF >30%), verapamil alone and with propranolol may cause mild to moderate depression of LV systolic function at rest, but not during exercise. This suggests verapamil does have the potential for deleterious effect, but probably only in CAD patients with considerably reduced EF at rest.

Collateral flow: Verapamil may reduce myocardial damage following coronary occlusion. To clarify the effects of verapamil on coronary and systemic hemodynamics, we determined dose-response curves in 8 closed chest dogs, beginning 5 minutes after coronary occlusion. Although verapamil increased blood flow to nonischemic myocardium (even in doses insufficient to decrease peripheral vascular resistance), collateral blood flow was unchanged. Thus, in closed chest dogs with acute coronary occlusion, verapamil does not alter perfusion of ischemic regions. If verapamil is capable of diminishing infarct size, then these results suggest that it does so through mechanisms other than improving collateral flow.

CORONARY ARTERY DISEASE

Indications for coronary artery bypass grafting (CABG) in patients with stable CAD

Attitudes regarding indications for CABG in patients with stable CAD are based on natural history studies derived largely from severely symptomatic patients studied in the late 1960s and early 1970s. These data indicate an approximately 6-11% annual mortality in patients with 2 or 3 vessel disease respectively. Since post-operative mortality averages about 2-5% per year, the case for operating on all patients with 2-3 vessel CAD becomes compelling. However, during the past 5 years we have evaluated patients with CAD who are asymptomatic, or who have only mild symptoms. Two or 3 vessel disease was present in 75% of the 137 patients followed. Overall annual mortality in these patients has been only 3%. Moreover, despite a history of mild symptoms, 32 (24%) of the patients had poor exercise capacity on objective testing (<100 watts on the bicycle). In these patients annual mortality was 5%, and 15% per year required operation for increased symptoms. Of patients who achieved 100 watts or more, annual mortality was only 1.5% and only 4%/year required operation because of progressive symptoms. LV EF, measured at rest and during exercise by radionuclide angiography, also aided in identifying those patients at higher and lower risk of either sudden death or progressive angina. No patient who manifested an increase in EF from rest to exercise died suddenly, even if exercise capacity was less than 100 watts. Moreover, in patients who increased EF and exercise to ≥ 100 watts, no deaths occurred and progressive angina developed in only 2%/year; in patients who did not increase EF and exercised to <100 watts, 6%/year died and 14%/year experienced

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progressive angina. Thus, a patient with CAD and no or mild symptoms, who demonstrates good exercise capacity and an increase in EF from rest to exercise, has an excellent prognosis (annual mortality <1.5%) even in the presence of 2 to 3 vessel disease. Given such results, it is highly unlikely that prophylactic CABG could enhance survival of this large group of patients with CAD.

Balloon Dilatation of the Coronary Arteries To date 31 patients have undergone balloon dilatation of the coronary arteries at the NIH. Single vessel CAD was present in 23 patients, 2 vessel disease in 7, and dilatation of a bypass graft was attempted once. Initial angiographic improvement was demonstrated in 19 (61%) patients. At subsequent follow-up of these 19 patients, 14 are improved and 5 developed severe symptoms with documented restenosis, all of the latter occurring within the first 3 months. Of patients deemed to have a successful angiographic result, all were free of angina following dilatation on exercise testing and all demonstrated an improvement in exercise EF and myocardial perfusion scan. Of the 5 patients who developed restenosis, 3 have undergone repeat angioplasty, 2 of whom improved and remain so; the third developed restenosis after the second procedure. Thus, we conclude balloon dilatation can be performed in selected CAD patients with many experiencing initial and medium term (6-12 months) angiographic and functional improvement.

Abnormalities in LV Diastolic Filling Abnormalities in diastolic filling have been recognized in patients with CAD, but the frequency of such abnormalities is unknown. To determine LV diastolic filling characteristics of patients with CAD, 221 patients were studied by radionuclide angiography. Abnormal LV filling at rest (reduced LV PFR or increased time to PFR) was found in 90% of all patients with CAD, 86% of patients with normal rest LVEF, and 85% of patients without ECG Q waves. Thus, diastolic filling is abnormal in most patients with CAD at rest, and is independent of abnormalities in LV systolic function.

Sensitivity and specificity of radionuclide cineangiography in CAD patients without prior infarction Radionuclide (RN) angiography is highly sensitive in detecting CAD. Many pts with CAD, however, have previous AMI with abnormal LVEF at rest and with Q waves on ECG. Inclusion of such patients in studies of LV function will enhance sensitivity figures. To assess sensitivity and specificity of RN cineangiography in detecting CAD in patients without prior MI, we studied 141 patients with CAD and normal rest EF, 123 patients without ECG Q wave, and 50 patients with chest pain and normal coronary angiograms. Sensitivity in patients without evidence of prior MI was 90%, with a specificity of 95%. When abnormalities in diastolic function were also analyzed, sensitivity increased to 99%, with specificity only decreasing to 90%. Thus, RN cineangiography is extremely sensitive and specific in detecting CAD, even in patients with normal rest LVEF and without prior MI.

NHLBI Type II Coronary Intervention Study The primary aim of this randomized, double-blind prospective study, carried out in collaboration with the Molecular Disease Branch, is to determine whether lowering LDL cholesterol with cholestyramine and diet in patients with premature CAD and Type II hypercholesterolemia, will retard the progression of CAD. The major criterion we will employ to answer this question is whether there is regression of anatomic disease or evidence of slower progression, conclusions that will be based on coronary angiograms obtained at initiation into study and after 5 years of treatment. By October 1, 1980, over 3/4 of the study participants will have completed their 5 year studies. All participants will have completed the study by the summer of 1981.

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Proteolysis and ischemic damage during acute myocardial infarction (AMI) Biochemical, ultrastructural, and immuno-histological evidence suggest that increased lysosomal hydrolysis may play a causal role in death of myocardial cells that otherwise may have survived an acute but transient ischemic insult. Furthermore, it has been suggested that interventions which stabilize lysosomal enzymes, and thereby interfere with proteolysis, have the potential to salvage ischemic myocardium that otherwise would become necrotic. However, the effect on proteolysis of AMI, produced in intact animals, has not been evaluated. We therefore determined the rate of proteolysis following acute LAD occlusion, assessed by determining the amount of tyrosine produced by ischemic and non-ischemic slices of myocardium. In both dogs and rats proteolysis was diminished during AMI. Moreover, we assessed the potential of leupeptin, an inhibitor of lysosomal and cytoplasmic thiol-proteases, to interfere with proteolysis and reduce the size of mycoardial infarction. In rats, leupeptin reduced total proteolysis in ischemic regions. Despite the reduction of proteolytic activity, however, infarct size was no smaller in those rats pretreated with leupeptin. We therefore conclude that proteolysis does not increase during acute myocardial infarction, and 2) although proteolysis still occurs during ischemia and can be inhibited by leupeptin, such inhibition does not reduce ultimate infarct size. These data suggest that cellular proteases do not cause cell death during AMI, making it unlikely that drug-induced inhibtion of proteolysis would prevent ischemic myocardium from becoming necrotic.

Determinants of arrhythmic death due to coronary spasm Coronary spasm may occur in the presence or absence of CAD, but whether CAD contributes to the likelihood of spasm causing sudden death is unknown. We determined the effect of pre-existing coronary stenosis on the incidence of ventricular fibrillation (VF) during reperfusion following coronary artery occlusion. In 20 open-chest dogs an occluder was placed on the circumflex coronary artery (CFX) to partially restrict blood flow. We completely occluded the CFX for 30 minutes then abruptly released it, leaving the partial occluder in place. During reperfusion, CFX flow was 84% of baseline in surviving dogs and 156% in dogs dying in VF ($p<.02$). In another 16 dogs, the LAD was gradually occluded by an ameroid constrictor; after 20 to 39 days, we abruptly occluded the CFX for 30 minutes, then abruptly reperfused. Collateral blood flow (CBF) to the circumflex bed (measured 10 minutes after occlusion) was 27% of normal zone flow in dogs dying in VF at reperfusion, versus 58% in dogs surviving ($p<.05$). Thus, reperfusion VF is greater in dogs 1) when sudden occlusion occurs in vessels with lesions producing minimal obstruction to baseline flow than when it occurs in vessels with more severe lesions, and 2) when sudden occlusions occur in normal vessels supplied with few collaterals from totally occluded feeder vessels, than when collateral flow is extensive. These results suggest that risk of VF during release of coronary spasm may actually be greater in patients without CAD than in those with CAD.

Inadequacy of coronary collateral blood flow reserve in myocardium surviving AMI During nontransmural AMI subendocardium within the distribution of an occluded coronary artery infarcts and subepicardium is spared. While CBF flow to surviving epicardium returns toward normal at rest, its capacity to respond to stress is unknown. Thus, we induced AMI in 9 awake dogs; 3 to 4 days later we measured the ability of spared epicardium to increase flow in response to pacing-induced stress. While flow increased in nonischemic epicardium from

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1.39 to 2.89 ml/min/g, flow increased in surviving epicardium from .78 to only 1.01 ml/min/g, indicating reduced reserve capacity for CBF in tissue surviving AMI. These data suggest the potential for stress to induce angina, arrhythmic death or extension of AMI across its transmural border zone in subjects with prior AMI.

Relation between Electrical Instability and Myocardial Infarction Size
No direct evidence exists relating size of AMI to electrical instability (EI) several days later. Moreover, it has been suggested that if ischemic myocardium is salvaged by intervention during AMI, the surviving myocardium is electrically unstable. Data supporting this hypothesis, however, are lacking. We therefore studied conscious dogs 3 to 4 days after AMI. Arrhythmic responses to ventricular electrical stimulation were determined, and a score for EI was assessed by the type of abnormal responses, the nature of the stimuli required to produce the responses (i.e., single or double stimuli), and the time in diastole at which they could be elicited. EI correlated directly with MI size (r= .96). Moreover, EI was less in those hearts with greater amounts of risk region or LV naturally salvaged (R = -.94, -.75). This was due mainly to the inverse relation between percentage LV infarcted and percent LV salvaged (R = -.87). We conclude that 1) EI determined by electrical stimulation several days post-MI increases in proportion to size of MI, and 2) LV salvaged by intervention may enhance rather than reduce electrical stability post-MI.

HYPERTROPHIC CARDIOMYOPATHY

Distribution of hypertrophy in patients with HCM HCM is a disease of cardiac muscle characterized by a hypertrophied nondilated LV. Morphologic distribution of the cardiomyopathic process was defined grossly by two-dimensional (2-D) echo in 125 patients and histologically in 52 patients at necropsy. 2-D echo showed hypertrophy to be widespread involving both the VS and anterolateral LV free wall in 54% of patients; other patients had hypertrophy confined to the VS or to the LV free wall. Patients with widespread hypertrophy had marked functional limitation more commonly (60%) than other patients (24%; p<.001). All 7 patients with cardiac arrest and subsequent survival had marked VS and LV free wall hypertrophy. While myocardial cell disorganization was most extensive in VS (mean area involved 36%), it was also marked in the LV free wall (26%). Marked cellular disorganization (>5%) was diffuse in 63% of 52 patients and was most extensive (p<.001) in 15 young (less than 25 years old) patients in whom sudden death was the first manifestation of disease. Hence, gross anatomic and histologic findings suggest the cardiomyopathic process in HCM is usually: 1) diffusely distributed throughout the LV (and not limited to VS); 2) is associated with marked functional limitation and premature sudden death.

Sudden death in HCM Sudden death is not a rare sequelae of patients with HCM, although the mechanism of these catastrophic occurrences is unknown. Our previous 24 hour ambulatory ECG monitoring study showed particularly high prevalance (66%) of high grade "malignant" (e.g., Lown Grade III or higher) ventricular arrhythmias in 100 patients with HCM. Because the significance of these arrhythmias in identifying patients at high risk for sudden death is not known, clinical outcome was followed prospectively. After 3 years, 15 patients underwent operation because of severe symptoms. Of the remaining 85, 58 (68%) had had high-grade arrhythmias. Five patients died during follow-up and in

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"malignant" arrhythmias were recorded. However, 54/58 (93%) of the patients with "malignant" arrhythmias survived the three-year follow-up period. We conclude that 1) high grade arrhythmias are commonly found in patients with HCM by ambulatory ECG monitoring; 2) although such arrhythmias appear to identify most patients with HCM at high risk of sudden death (e.g., high sensitivity) most patients with such arrhythmias do well (low specificity); 3) therefore, other (as yet undefined) factors must be present for ventricular instability in patients with HCM to evolve into terminal arrhythmias.

MOLECULAR CARDIOLOGY

Reversible phosphorylation of the contractile protein myosin constitutes one of the major regulatory systems in smooth muscle and non-muscle cells. When myosin from smooth muscle and vertebrate non-muscle cells is phosphorylated it can interact with actin and smooth muscle contraction or the equivalent in non-muscle cells (motility, cytokinesis, platelet secretion etc.) can take place. The section on Molecular Cardiology has continued its studies in this area, emphasizing during the past year a number of different aspects.

Smooth muscle and platelet myosin kinases and phosphotases Phosphorylation plays an important role in regulating smooth muscle contraction, as well as the contractile process in non-muscle cell. Two major muscle proteins undergo phosphorylation: myosin and myosin light chain kinase. Phosphorylation of smooth muscle myosin, as well as myosin isolated from non-muscle cells, is a prerequisite for actin-activation of the myosin ATPase activity. Myosin which is not phosphorylated cannot undergo actin-activation and therefore cannot undergo contraction.

The enzyme which catalyzes phosphorylation of smooth muscle and platelet myosin (myosin kinase) is inactive in the absence of Ca^{2+} and the calcium binding protein, calmodulin. Smooth muscle contraction (and an equivalent process in non-muscle cells) is initated by a rise in Ca^{2+} which binds to calmodulin. The Ca^{2+} calmodulin complex binds to and activates myosin kinase, which catalyzes phosphorylation of myosin, resulting in actin-activation of the myosin ATPase activitiy.

Phosphorylation of the enzyme myosin kinase by cyclic AMP dependent protein kinase results in a decrease in myosin kinase activity. It does this by weakening the binding of myosin kinase for calmodulin. Since a rise in cyclic AMP activates protein kinase, phosphorylation of myosin kinase in smooth muscle and platelets may constitute one pathway through which a rise in cyclic AMP would result in smooth muscle relaxation and the equivalent effect in platelets.

This process is reversible and recently we have isolated and purifed from smooth muscle two of the enzymes active in reversing the phosphorylation of myosin. One of these enzymes also catalyzes the dephosphorylation of myosin light chain kinase and restores the tight binding of calmodulin to myosin kinase, therefore restoring myosin kinase to full activity.

Antibodies to purified smooth muscle myosin light kinase isolated from turkey gizzards were developed in rabbits. Some of the antibodies were shown to inhibit kinase activity and were further purified by affinity chromatography on a column β of myosin kinase coupled to Sepharose. The antibodies are being used to study the role of myosin kinase in regulating contraction-relaxation in smooth muscle and nonmuscle cells.

Myosin phosphorylation has also been shown to play a role in regulating contraction in striated muscle. In one of these muscles found in the tail of the horseshoe crab (Limulus), phosphorylation is a dominant regulatory mechanism. We are preparing fragments of myosin from both Limulus and gizzard muscle in order to decipher exactly what step in the contractile process is affected by myosin.

Two important aspects of cardiac muscle contractile proteins are under study: 1) myosin and the other major contractile proteins from human hearts are being characterized by pyrophosphate gel electrophoresis, isoelectric focusing and peptide maps. These results will be compared to those obtained from similar studies with cardiac muscle from patients with hypertrophic cardiomyopathy (a genetic disease) as well as other diseases of cardiac hypertrophy. Recent work has indicated that cardiac myosin and possibly actin exists as isozymes and the purpose of these studies is to see if different isozymes may be expressed differently in various diseases; 2) canine cardiac myosin kinase is being characterized from dog hearts. To date we have succeeded in isolating what appears to be a fragment of the enzyme that retains its calmodulin binding site. The cardiac kinase, unlike the smooth muscle kinase, undergoes a Ca^{2+} calmodulin-dependent autophosophorylation, the significance of which is presently under study. The purpose of these studies is to understand how stimulation of the β-receptor in cardiac muscle results in an increase in contractile activity.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01661-05 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Natural History of Asymptomatic or Mildly Symptomatic Patients with Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Carolyn J. Ewels	Research Assistant	CB	NHLBI
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .5	PROFESSIONAL: .4	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients who have mild symptoms of angina pectoris with coronary artery disease and who have good exercise tolerance appear to have an excellent prognosis when followed on a medical program. Coronary revascularization as a prophylactic procedure to enhance survival in such patients does not seem necessary.

Project description: Coronary revascularization operation is often recommended to patients with coronary artery disease who are not severely symptomatic on the assumption that it will improve survival. The natural history studies on which this hypothesis is based were performed on patients studied in the 1960's; most of whom were severely symptomatic. In an attempt to define the natural history of patients, all asymptomatic or mildly symptomatic patients (pts) on medical management with coronary artery disease (CAD) admitted to NIH have been followed prospectively for 6 to 58 months (avg. 24). Pts with left main CAD were excluded. One hundred thirty eight pts have been followed. Double or triple vessel disease (D or TVD) was present in 75%. Ejection fraction was $\geq$55% in 69% of patients. During follow-up there have been 6 deaths with an annual mortality of (3%) in the whole group as well as those with D or TVD. Nineteen of 132 survivors had increased symptoms requiring coronary revascularization 2 to 48 months (avg 22) after entry. Remaining pts are symptomatically stable. Despite a history of mild symptoms, 32 (24%) of the pts could not achieve 100 watts on exercise testing. Of these patients, 41% either died (10%, annual mortality =5%) or required operation for increased symptoms (31%). Of pts who achieved $\geq$ 100 watts, only 11% ($p<.04$) either died, (3%, annual mortality =1.5%) or required operation for increased symptoms (8%). Thus, patients with no or mild symptoms, and with good exercise tolerance, appear to have an excellent prognosis despite double or triple vessel disease D. Thus, it would appear reasonable to manage these patients medically until more limiting symptoms develop.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01665-05 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Smooth Muscle Myosin Light Chain Kinase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mary Anne Conti	Graduate Student	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	William Anderson, Jr.	Chemist	CB	NHLBI
	J. Maurice Miles	Medical Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Myosin light-chain kinase can be phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase. Phosphorylation of myosin kinase decreases the affinity of this enzyme for the calcium-binding protein calmodulin by twenty fold. Dephosphorylation of myosin kinase by a phosphatase purified from smooth muscle reverses this effect and increases calmodulin binding to myosin kinase. The ability of cyclic AMP-dependent protein kinase to decrease the activity of myosin light chain kinase, provides a direct mechanism for cyclic AMP mediated smooth muscle relaxation.

PHS-6040
(Rev. 10-76)

Project description: Phosphorylation of the 20,000 dalton light chain of smooth muscle myosin by the specific enzyme, myosin light chain kinase, is required for actin-activation of myosin Mg-ATPase activity. The purified kinase (130,000 daltons), which shows a single band in SDS and urea polyacrylamide gel electrophoresis has a specific activity of 30 mol PO_4 transferred to the light chain/mg kinase/min at 24°. Enzymmatic activity is independent of cAMP but is completely dependent on $Ca^{2+}(10^{-6}M)$ and on the presence of the ubiquitous calcium-binding protein, calmodulin (CDR).

When myosin light chain kinase is incubated with the catalytic subunit of cAMP-dependent protein kinase in the absence of bound Ca^{2+}-calmodulin complex, one mole of phosphate (from Mg-ATP) can be incorporated per mole of myosin kinase. Phosphorylation of myosin light chain kinase decreases the rate at which the kinase phosphorylates myosin light chains by lowering the affinity of the kinase for its activator, calmodulin. The phosphorylated kinase has a 20-fold weaker binding constant for calmodulin than the unphosphorylated kinase (Ka2.5x 10^{-8} and 1.2x10^{-9}M, respectively). Phosphorylation of the kinase also decreases by 50% the maximal rate (Vmax) of enzymmatic activity. When phosphorylated myosin kinase is dephosphorylated by incubation with smooth muscle phosphatase I (see Z01 HL 01752-01CB) the affinity of the kinase for calmodulin increases to 10^{-9}M, a binding constant equivalent to that of the unphosphorylated kinase. The Ca^{2+}-dependence of calmodulin binding to and activation of myosin light chain kinase will be investigated by Ca^{2+}titration of the phosphorylated and unphosphorylated kinase at various calmodulin concentrations.

The relationship between the site of calmodulin-binding and the active site on myosin kinase was investigated by brief tryptic digestion of the kinase in the presence or absence of calmodulin. Digestion of the kinase in the absence of bound calmodulin decreases total kinase activity and generates a kinase which no longer requires calcium for activity. In contrast, digestion of the kinase in the presence of calmodulin protects total enzymmatic activity and partially preserves calcium dependence.

Publications: Adelstein, R.S., Conti, M.A., Hathaway, D.R. and Klee C.B.: Phosphorylation of smooth muscle myosin light chain by the catalytic subunit of adenosine 3':5'-monophosphate dependent kinase. In Usdin E, Kopin, I.J. and Barchas J. (Eds.): Catecholamines: Basic and Clinical Frontier New York, Pergamon Press, 1979, pp 992-994.

Conti, M.A. and Adelstein, R.S.: Phosphorylation by cyclic 3':5' -monophosphate-dependent protein kinase regulates myosin light chain kinase. Fed. Proc. 39, 1564-1573, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01670-04 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Natural History of Aortic Regurgitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Carolyn Ewels	Research Assistant	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .2	PROFESSIONAL: .1	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sixty-nine patients with varying degrees of aortic regurgitation in either NYHA Functional Class I or II have been evaluated and are being followed prospectively in order to better assess the natural history of this disease. Admission studies included echocardiography, rest and exercise radionuclide angiography, measurement of pulmonary artery wedge pressure during exercise, and 24-hour ambulatory monitoring. These studies should allow us to identify more sensitive indicators of impending clinical deterioration than are presently available.

Project description: In patients with aortic regurgitation (AR), we presently employ the development of symptoms as the primary indication for proceeding with valve replacement. However, the results of operation are often less than optimal in terms of symptom relief and long-term survival. In order to develop more sensitive indicators of deterioration of cardiac function in asymptomatic patients with AR, extensive clinical and hemodynamic evaluations are being performed. To date, 77 functional class I or II patients with aortic regurgitation have been evaluated with echocardiograms, graded treadmill exercise tests, radionuclide cineangiograms at rest and during supine exercise, measurement of pulmonary artery wedge pressure response to intense supine exercise and 24-hour ambulatory monitoring. The average follow-up time has been 26 months. This group has been divided into 18 asymptomatic patients with mild aortic regurgitation, 49 asymptomatic patients with moderate to severe AR, and 10 patients who have undergone aortic valve replacement after having been initially followed in the Natural History Study. These latter ten were operated on either because of decreasing left ventriclular function or the development of symptoms while under observation. Only one patient from this group has died at this point, and he had mild AR, but significant coronary artery disease at the time of post-mortem examination.

In comparison to this group, the same intensive evaluation has been carried out on 35 patients who were referred either with moderate to severe aortic regurgitation and significant symptomatology or evidence of left ventricular dysfunction. These patients have had their aortic valves replaced and are being re-evaluated six months after operation. There have been five deaths in this group. Three were at the time of operation and two occurred within the first month of discharge. Both of these latter patients had also undergone aortic root replacement for aortic root disease.

Since the study has been initiated, we have already revised our indications for valve replacement. This change in policy has resulted not only from the data from the present study, but also from data obtained on patients evaluated prior to initiations of this study. Valve replacement is performed not only in patients with significant symptomatology, but also when left ventricular systolic function is compromised, as evidenced by echocardiography (left ventricular systolic dimension greater than 55 mm).

Exercise radionuclide angiography and exercise-induced changes in pulmonary capillary wedge-pressure also are measurements capable of distinguishing the three groups of mildly or asymptomatic patients listed above. Since only ten patients have crossed over from the medical to surgical groups, we have not as yet been able to establish specific criteria applicable to these tests which will be helpful in predicting clinical or hemodynamic deterioration. However, it is clear that hemodynamic measurements made during intense exercise are not sensitive indicators of left ventricular function and these measurements are no longer being made.

Significant high grade ventricular ectopy has been detected in this group, but its significance is uncertain. With 24 hr ambulatory monitoring, 31/69 pts with AR with no or mild symptoms had Lown class 4A or 4B ventricular ectopy while on no antiarrhythmic treatment. Average follow-up was 21±1 mo. Ventricular ectopy was not related to severity of AR or presence of coronary disease (CAD). In 29/39 additional consecutive pts studied prior to aortic valve replacement high grade ventricular ectopy was also found. Ventricular tachycardia was present in 16 non-operative patients and 17 operative pts (NS). Rx was initiated in only 4 NOP pts with ventricular tachycardia. Non-operative pts without ventricular tachycardia were not placed on Rx and high grade ventricular ectopy continued in 16/20 pts who underwent repeat ambulatory monitoring. Six mo after AVR, ambulatory monitoring was repeated in 30 pts and 29 continued to have hi grade ventricular ectopy. In 16 operative pts with ventricular tachycardia, 6 had ventricular tachycardia after aortic valve replacement and 4 had 4A ventricular ectopy. Only one non-operative pt died (ventricular tachycardia, CAD). Three pts died at the time of aortic valve replacement (2=high grade ventricular ectopy, 1 = CAD, high grade ventricular ectopy). No other pts have died after aortic valve replacement with a mean follow-up time of 20±2 mo. These data indicate that high grade ventricular ectopy is common in mildly or severely symptomatic pts with AR and occurs independently of the presence of CAD. However, high grade ventricular ectopy has not been associated with increased mortality during follow up of either non-operative patients or operative patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01672-04 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Maintenance of a Computerized Clinical Data Bank for Cardiology Patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Charles McIntosh	Senior Surgeon	SU	NHLBI
	Gerald D. Stoner	Head, Applied System Program Section	DMB	DCRT
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Surgery Branch, NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.1	.9

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A computerized clinical data bank has been established for all cardiology patients. Inpatient and outpatient data will include symptom description, and X-ray, ECG, catheterization, echocardiogram, and radionuclide angiogram results.

PHS-6040
(Rev. 10-76)

Project description: A computerized data bank has been established for cardiology patients in order to provide easy access to patient data for clinical and investigative purposes. Included in the data base is information from both outpatient and inpatient visits as well as the identification of procedures and diagnoses generated at other institutions. The data includes symptom description, X-ray, ECG, catheterization, radionuclide, and echocardiogram interpretations, listing of current medications, and disposition of the patient. In addition, with the assistance of the "MIS" system, a printout of the catheterization results is generated for the medical records. Data accumulation was begun on January 1, 1978 and current and retroactive information has been entered on all of our patients. In the winter of 1979, the data base of the Cardiology Branch and the one already in service under the direction of Dr. Charles McIntosh of the NHLBI Surgical Branch were merged into one system, thus facilitating access to data on all ptients with cardiovascular disease. Queries of the data base for clinical and research purposes have averaged about 10 per month. A summary of available "pertinent" clinical data is now obtained for each outpatient visit to the Cardiology and Surgery Clinics in order to make chart reviews easier at these visits.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01689-04 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Phosphorylation of Cardiac Myosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lydie Rappaport	Expert	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	C. Robert Eaton	Physicist	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.2	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A fragment of the enzyme myosin light chain kinase has been isolated from canine cardiac muscle. This fragment has two important properties: 1) it requires Ca calmodulin to phosphorylate myosin light chains; 2) it undergoes a Ca calmodulin-dependent autophosphorylation.

PHS-6040
(Rev. 10-76)

Project description: Cardiac muscle myosin light chain kinase has been purified from dog heart ventricles. The final step of purification is chromatography on an affinity column of calmodulin coupled to Sepharose-4B. The molecular weight of the purified kinase is 56,000 in SDS-PAGE and its activity for transferring phosphate to the 20,000 dalton light chain of myosin is completely dependent on Ca and calmodulin. Since Walsh et al. have previously reported that cardiac muscle myosin kinase has a molecular weight of 85,000 (J. Biol. Chem. 254, 12136-12144, 1979) the enzyme reported on here may be an active fragment of a larger molecule. The 56,000 dalton myosin kinase undergoes an autophosphorylation which is dependent on the presence of both Ca and calmodulin. The rate of autophosphorylation is extremely rapid, being complete in a few minutes at 0°, with 1 mole of phosphate incorporated per mole of kinase. The rate of autophosphorylation was independent of enzyme concentration suggesting an intramolecular mechanism. Autophosphorylation results in a 50% reduction in myosin kinase activity when assayed with smooth muscle myosin light chains.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01712-03 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Ca^{2+} -dependent Enzymes from Human Platelets

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert S. Adelstein	Head, Molecular Cardiology CB NHLBI
Other:	Robert Eaton	Physicist

COOPERATING UNITS (if any)

David R. Hathaway, M.D.	Indiana University School of Medicine

LAB/BRANCH
Cardiology Branch

SECTION
Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.2	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The project involves two different platelet enzymes: 1) Platelet myosin light chain kinase: Phosphorylation of this enzyme by cAMP-dependent protein kinase decreases the activity of this enzyme. This might explain one way in which a rise in cAMP would act to inhibit platelet release 2) Platelet protease: A Ca -dependent protease which degrades human platelet myosin and smooth muscle myosin kinase has been purified. This protease might play an important role in the turnover of platelet contractile proteins.

PHS-6040
(Rev. 10-76)

Project description: Platelet myosin light chain kinase was purified in a Ca^{2+}-calmodulin dependent form by including a number of protease inhibitors. The platelet myosin kinase is a substrate for cyclic AMP-dependent protein kinase. One mole of phosphate is incorporated per mole of kinase. Phosphorylation of platelet myosin kinase results in a decrease in the activity of this enzyme. This decreased activity might explain one way in which a rise in the level of cyclic AMP might regulate platelet function.

A Ca^{2+}-dependent protease, which degrades platelet myosin as well as smooth muscle myosin light chain kinase, has been purified and characterized. The enzyme has a molecular weight of 83,000 and is dependent on the presence of Ca^{2+}, but not calmodulin, for activity.

Publications: Adelstein, R.S. and Hathaway, D.R.: Role of calcium and cyclic adenosine 3':5' monophosphate in regulating smooth muscle contraction Am. J. of Cardiol., 44: 783-787, 1979

Hathaway, D.R., Eaton, C.R. and Adelstein, R.S.: Regulation of human platelet myosin kinase by calcium-calmodulin and cyclic AMP. In: Proceedings of the International Workshop on Coagulation Amsterdam, Elsevier/North Holland, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01719-03 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Antiarrhythmic Effects of Verapamil in Patients with Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .1	PROFESSIONAL: .05	OTHER: .05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil has been successful in treating paroxysmal atrial tachycardia in patients with hypertrophic cardiomyopathy as well as other forms of heart disease. It has not been of any consistent benefit in patients with significant ventricular arrhythmias.

PHS-6040
(Rev. 10-76)

Project description: Verapamil, a "calcium antagonist", is an effective antiarrhythmic agent for supraventricular tachycardias, although less success has been obtained with ventricular arrhythmias. Patients with hypertrophic cardiomyopathy tolerate all forms of tachyarrhythmias very poorly, and ventricular or supraventricular tachyarrhythmias may be a major mechanism for sudden death in this group.

It is well known that verapamil can acutely revert supraventricular tachycardia (SVT) to sinus rhythmn in 80-100% of patients. However, the use of oral verapamil to maintain sinus rhythm in these patients has not been well studied. In five patients (three without other demonstrable cardiac disease, who were refractory to the usual treatment for SVT, we have been able to eliminate or markedly reduce the episodes of SVT with the chronic use of verapamil. These patients have been on the drug for periods up to 28 months.

Verapamil administration has been especially helpful in patients with hypertrophic cardiomyopathy who are in atrial fibrillation. We have initiated verapamil treatment in 13 such patients and 11 have had an excellent clinical response for periods up to 18 months. Three of these patients have surprisingly reverted to sinus rhythm and have remained in this rhythm for periods up to six months.

Although two patients with significant (Lown Grade III-IV) ventricular arrhythmias have had a ≥75% reduction in ventricular ectopy with verapamil, many others have had no improvement in their ventricular arrhythmias and have required additional antiarrhythmic therapy.

In summary, verapamil seems to be a very effective antiarrhythmic agent for patients with hypertrophic cardiomyopathy who suffer from atrial tachyarrhythmias. This observation is especially important in those patients with chronic atrial fibrillation who previously required digoxin to slow the ventricular response, since the positive inotropic effect of digitalis glycosides can increase left ventricular outflow obstruction in patients with obstructive hypertrophic cardiomyopathy. The medication does not seem to be as helpful in patients with ventricular arrhythmias, but an evaluation on an individual basis is recommended, for verapamil has been effective in suppressing ventricular arrhythmias in a few patients.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01720-03 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hemodynamic Effects of Verapamil in Patients with Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .2	PROFESSIONAL: .1	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil, a calcium antagonist, reduced left ventricular outflow obstruction in patients with hypertrophic cardiomyopathy, and produced no adverse effects on left ventricular function. In order to evaluate the mechanism by which the drug works, evaluation of its effect on diastolic events is presently being undertaken.

Project description: Various investigators have proposed that abnormal Ca metabolism may contribute to the pathologic and physiologic abnormalities in some experimental cardiomyopathies. To help assess this possibility, the Ca antagonist verapamil was infused in 27 patients with hypertrophic cardiomyopathy (HCM) at the time of catheterization. Basal and provoked left ventricular outflow tract gradients were measured under control (C) conditions and during 3 progressively greater doses of verapamil (0.007, 0.014, 0.021 mg/kg/min). The peak verapamil effects on left ventricular outflow tract gradients in patients who had a gradient of at lest 30 mmHg in the C state were: (mean ±SEM).

	Basal	Valsalva	Amyl Nitrite	Isoproterenol
Control	84 ± 6	90 ±8	82 ± 9	106 ± 14
Verapamil	56 ± 7	64 ± 10	43 ± 11*	63 ± 5*

Heart rate (HR), systolic blood pressure (SBP), mean pulmonary artery wedge pressure (PCW), and cardiac index (CI) were also measured before and during the 3 verapamil doses (V1-V3).

HR	76 ± 3	77 ± 2	81 ± 3*	81 ± 6
SBP	116 ± 4	108 ± 3*	107 ± 4*	99 ± 5*
PCW	14 ± 1	13 ± 1	15 ± 1	15 ± 1
CI	2.6 ± 0.1	2.7 ± 0.2	3.0 ± 0.3	2.8 ± 0.2

= $p<.05$ * = $p<.005$ (compared to C)

In order to determine the mechanism by which the drug works in this disorder, its effect on the left ventricular pressure-volume relationship is presently being evaluated using a micromanometer tip catheter and radio isotope imaging. Preliminary results indicate an abnormal left ventricular pressure-volume relationship which is improved by intravenous verapamil administration.

Verapamil is also being administered intravenously in the catheterization laboratory to patients with high left ventricular filling pressure and/or the non-obstructive form of the disorder in order to determine its effect in selected high risk patients who are not operative candidates.

These results indicate that verapamil can significantly decrease left ventricular outflow tract obstruction and has a beneficial effect on diastolic function in patients with HCM.

Publications: Rosing, D.R., Kent, K.M., Borer, J.S., Seides, S.F., Maron, B.J. and Epstein, S.E.: Verapamil Therapy: A New Approach to the Pharmacologic Treatment ofHypertrophic Cardiomyopathy: I. Hemodynamic Effects. Circulation 60: 1201-1207, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01727-03 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Septal Disorganization as Specific Marker for Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Noriko Sato	Guest Worker	CB	NHLBI
	William C. Roberts	Chief, Pathology Branch	PB	NHLBI
	Jesse E. Edwards	Chairman, Dept. of Pathology	United Hosp.-Miller Div.	
	Roma S. Chandra	Staff Pathologist	Children's Hosp. Med. Center	

COOPERATING UNITS (if any)

Pathology Branch, NHLBI
United Hospital - Miller Division, St. Paul, Minn.
Children's Hospital Medical Center, Washington, D.C.

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.3	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Marked disorganization of cardiac muscle cells in the ventricular septum is a highly specific (as well as sensitive) marker for hypertrophic cardiomyopathy. Although septal disorganization may occur in patients with aortic or pulmonic atresia (as well as other congenital heart malformations) the areas of septum involved are usually extremely small.

PHS-6040
(Rev. 10-76)

Project description: The presence of numerous abnormally arranged cardiac muscle cells in the ventricular septum has been considered to be a characteristic anatomic feature of patients with hypertrophic cardiomyopathjy. However, it has been suggested that the ventricular septum of infants with certain congenital cardiac diseases (such as aortic or pulmonic valve atresia) contains disorganized cardiac muscle cells similar to that present in patients with hypertrophic cardiomyopathy. To test the validity of this concept and the true specificity of septal disorganization for hypertrophic cardiomyopathy, sections of ventricular septum were obtained at necropsy from 276 patients and the extent of ventricular septal disorganization was determined quantitatively. Disorganization was most marked in the infants, children and adults with hypertrophic cardiomyopathy - i.e., present in 95% of 60 patients; the mean area of septum disorganized was 31 ± 3%. Although disorganized cells were present in 64% of 33 infants with aortic or pulmonic valve atresia, these cells occupied extremely small areas of ventricular septum (mean area of septum disorganized 2.8 ± 0.7%; $p<0.001$). Furthermore, the minimal septal disorganization present in aortic or pulmonic valve atresia was similar to that found in 91 infants with other congenital heart malformations (mean area disorganized 1.4 ± 0.6%), and in 92 normal fetuses or infants (mean area disorganized 0.3 ± 0.1%). Hence, extensive ventricular septal disorganization is a highly sensitive and specific finding for hypertrophic cardiomyopathy, although small areas of disorganization may occur in infants with a variety of other heart diseases, including aortic or pulmonic valve atresia.

Publications: Maron, B.J., Sato, N., Roberts, W.C., Edwards, J.E. Chandra, R.S.: Quantitative analysis of cardiac muscle cell disorganization in the ventricular septum: Comparison of fetuses and infants with and without congenital heart disease and patients with hypertrophic cardiomyopathy. Circulation 60: 685-696, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01744-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Effect of Nitroglycerin in Patients with Aortic Regurgitation and LV Dysfunction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine Department, CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.05	.04	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nitroglycerin (TNG) was administered to 15 patients with aortic regurgitation and left ventricular (LV) dysfunction determined by radionuclide cineangiography during supine exercise. TNG resulted in a 25% decrease in LV end-diastolic volume (LVEDV) during exercise ($p<.01$) with no change in exercise heart rate; TNG also resulted in a significant increase in exercise LV ejection fraction (40±10% pre TNG to 47±10% post-TNG). Thus, despite reduction in LVEDV (causing reduction in wall stress), stroke volume diminished only 5% at maximal exercise. The TNG-induced reduction in LV wall stress with maintenance of external work may be of value in the long-term management of patients with aortic regurgitation.

PHS-6040
(Rev. 10-76)

Project description: Previous studies have demonstrated that vasodilators can reduce regurgitant fraction at rest in pts with aortic regurgitation. To determine the effect of nitroglycerin (TNG) in pts with aortic regurgitation and depressed left ventricular (LV) function, we utilized radionuclide cineangiography during exercise before and after TNG administration in 15 pts. Prior to TNG, 5 pts had subnormal LV ejection fraction (EF) at rest (<45%), and all had subnormal LVEF during exercise (<55%). After TNG in doses sufficient to diminish systolic arterial pressure ≥10 mm Hg and/or increase heart rate ≥10 beats/min, LV end- diastolic volume fell 18% at rest and 25% during exercise (both $p<.01$ compared with pre-TNG values). TNG caused no change in resting LVEF (50±7% pre-TNG vs 53±9% post-TNG, N.S.); however, LVEF increased significantly during exercise after TNG (40±10% pre-TNG to 47±10% post-TNG, $p<.001$). Exercise heart rates were unchanged. Despite reduction in LV end-diastolic volume (causing reduction in wall stress), stroke volume diminished only 5% at maximal exercise. The TNG-induced increase in LVEF during exercise did not correlate with the level of LVEF at rest or during exercise, with resting hemodynamic values, or with echo LV diastolic or systolic dimensions or percent fractional shortening. Hence, TNG produces a decrease in LV end-diastolic volume and an increase in LVEF during exercise in pts with aortic regurgitation that is independent of the degree of LV dysfunction. The TNG-induced reduction in LV wall stress with maintenance of external work may be of value in the clinical management of pts with aortic regurgitation and impaired LV systolic function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01749-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Long-term Hemodynamic Assessment of the Porcine Heterograft in the Mitral Position: Late Development of Valvular Stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lewis C. Lipson	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Section C-V Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Charles L. McIntosh	Senior Surgeon	SU	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	Andrew G. Morrow	Chief, Surgery Branch	SU	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.3	.7

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Although porcine heterografts are widely used for mitral valve replacement, there are few data on the long-term durability of this prosthesis. We found an appreciable incidence of acquired prosthetic valve stenosis in clinically stable patients who had porcine heterografts in place for more than five year.

PHS-6040
(Rev. 10-76)

Project description: Porcine heterografts are widely used for mitral valve replacement, although concern has arisen about the long term durability of these valves. To elucidate further the long term hemodynamic performance of porcine heterografts, we undertook a study of patients who had porcine mitral valves in place for more than five years and who had *no* clinical signs or symptoms suggestive of valve dysfunction. Of the first 54 patients who had porcine valves implanted in the mitral position, 18 were available for catheterization; all had a routine hemodynamic study shortly postoperatively (mean 7 months) for comparison. Mean follow up was 85 months (range 61 to 111). Compared to the early postoperative data, there was a significant increase in mean mitral valve gradient from 5.9 ± 0.7 to 8.6 ± 0.7 mm Hg ($p<0.01$), and a significant decrease in calculated mitral valve area from 2.2 ± 0.2 to 1.7 ± 0.2 cm ($p<0.01$). Moreover, a decrease in valve area greater than 0.5 cm was seen in 7 patients; 5 of 7 patients with valves in place for greater than 80 months showed such a decrease, compared to only 2 of 11 patients with valves in place for 80 months or less ($p<0.05$). We conclude that there is a significant incidence of hemodynamic deterioration of porcine heterografts in the mitral position for greater than five years, even in patients who are clinically stable. Whether the higher incidence of valve deterioration in the valves in place longer is due to faulty early manufacture or surgical techniques, or whether it represents progressive valve degeneration, remains to be seen. These date must be weighed against the generally favorable clinical performance of porcine heterografts when the choice of mitral valve prosthesis is made.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01750-02 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Verapamil: A New Approach to the Treatment of Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
Other:	John R. Condit	Research Assistant	CB	NHLBI
	Barry J. Maron	Senior Investigator	CB	NHLBI
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .5	PROFESSIONAL: .4	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil was administered to 78 patients with hypertrophic cardiomyopathy in order to try to improve their style of living. Forty-two have remained on the medication for at least six months (range 6-30). These patients have manifested an improvement in subjective symptomatic status as well as exercise capacity over this period of time.

154

PHS-6040
(Rev. 10-76)

Project description: The primary approach to symptomatic therapy in patients with hypertrophic cardiomyopathy (HCM) is the use of beta blocking agents. We previously demonstrated that verapamil reduces left ventricular outflow tract obstruction and improves exercise capacity and subjective symptomatology in hospital in such patients. To assess the chronic effects of verapamil in HCM, 78 patients whose lifestyle was unacceptable despite propranolol therapy were begun on oral verapamil in hospital between September, 1977 and September, 1979. Ten patients were not discharged on verapamil due to: side effects 5 (1 death), non-compliance 2, and symptomatic deterioration 3. The drug was stopped in 26/68 patients discharged on verapamil; in 20 because symptoms were unrelieved or recurred, in three because of an inability to comply with the protocol and in 1 because of drug side effects. Two patients died while on chronic drug treatment. Forty-two patients have remained on verapamil (6-30 mos; median = 14). Treadmill exercise duration was evaluated in 41 of the 42. Long-term verapamil led to improved exercise duration compared to in-hospital a) control (+53±10%; $p<.001$), b) propranolol (+ 32±12%; $p<.01$), c) verapamil (+18±7%, $p<.25$). Adverse hemodynamic effects experienced in the 78 patients included three deaths, five episodes of pulmonary congestion, three of hypotension, two cases of sinus arrest, five of development of junctional rhythm and 3 of Wenkebach 2° heart block. Non-cardiovascular side effects included upper abdominal discomfort in two patients, frequent constipation, and occasional descriptions of hair loss. In almost all cases of hypotension, junctional rhythm, 2° heart block, and non-cardiovascular problems, the drug was continued at reduced dosage, although therapeutic efficacy was sometimes compromised because of an inability to use higher doses. Of the 78 patients started on verapamil in hospital, 36 (46%) have either improved their functional class by at least 1 grade and/or have increased their exercise duration by 15%. Thus, verapamil appears to offer a much needed new therapeutic approach to the treatment of HCM.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01752-02 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Purification of Myosin Light Chain Phosphatases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mary Pato	Fogarty Fellow	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	William Anderson	Chemist	CB	NHLBI
	J. Maurice Miles	Medical Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.2	OTHER: 0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two different phosphatases have been isolated from smooth muscle. One phosphatase is a trimer and dephosphorylates smooth muscle myosin as well as myosin light chain kinase. The second phosphatase, which depends on Mg for its activity, only dephosphorylates myosin light chains and not myosin kinase.

Project description: The actin-activation of the ATPase activities of myosins isolated from various smooth muscle and non-muscle cells require phosphorylation of their 20,000 dalton light chains by myosin kinase. The latter enzyme can be phosphorylated by cAMP dependent protein kinase resulting in a decrease of its activity. In an attempt to attain a better understanding of the regulation of contraction in smooth muscle, the myosin phosphatases in turkey gizzard smooth muscle were studied.

Two different myosin phosphatases were purified from turkey gizzards by column chromatography using Sephacryl S-300, DEAE-Sephacel and aminohexyl-Sepharose resins. The final step of purification was an affinity column of thiophosphorylated light chains coupled to Sepharose 4B. Myosin phosphatase I is a trimer with a molecular weight of 165,000 ± 5,000 as determined by sedimentation equilibrium. On SDS-polyacrylamide gels,the denatured enzyme exhibits three polypeptides bands with molecular weights of 60,000, 55,000 and 38,000. Myosin phosphatase II is a single polypeptide of 43,000 (or a multiple of it) as shown by SDS-polyacrylamide gel electrophoresis. In addition to differences in molecular weight and subunit composition, the two enzymes differ in other properties. Myosin phosphatase I dephosphorylates myosin light chains and myosin kinase at comparable rates while myosin phosphatase II preferentially dephosphorylates myosin light chains. Mg^{2+} is required for maximal activity of phosphatase II while phosphatase I has no metal ion requirement for activity. The pH profiles of the two enzymes are different as well as the effect of NaF on their activities. Various factors were observed to inhibit the activity of phosphatase I. Some of these factors are: increasing ionic strength, phosphate, pyrophosphate, ATP and ADP.

We are currently pursuing a study of the regulation of these myosin phosphatases and their physiological role in the contraction of smooth muscle.

Publications: Adelstein, R.S., Pato, M.D. and Conti, M.A.: Regulation of smooth muscle myosin kinase activity by phosphorylation and dephosphorylation. In Symposium on Regulatory Mechanism of Muscle Contraction Tokyo, 1979 (in the press)

Adelstein, R.S., Pato, M.D., Rappaport, L., Conti, M.A., Hathaway, D.R. and Eaton, C.R., Regulation of contractile proteins by kinases and phosphatases In. FMI-EMBO Workshop on Protein Phosphorylation and Bioregulation Basel, Switzerland S. Karger, A.G., 1980 (in the press)

Pato, M.D. and Adelstein, R.S. (1980) J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01761-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Percutaneous Transluminal Coronary Angioplasty

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	K. M. Kent	Head, Cardiovascular Diagnosis	CB NHLBI
Other:	D. R. Rosing	Senior Investigator	CB NHLBI
	L. C. Lipson	Senior Investigator	CB NHLBI
	R. O. Bonow	Senior Investigator	CB NHLBI
	C. McIntosh	Senior Surgeon	SU NHLBI
	M. Jones	Senior Surgeon	SU NHLBI
	S. Bacharach	Physicist	NM CC
	M. Green	Chief, Applied Physics Sec.	NM CC
	S. Epstein	Chief, Cardiology Branch	CB NHLBI

COOPERATING UNITS (if any)

Surgery Branch, Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .5	PROFESSIONAL: .4	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Dilatation of narrowings of the coronary arteries has been performed in patients during a procedure similar to cardiac catheterization. The narrowings of the coronary arteries can be successfully dilated in some patients and this initial dilatation leads to long-lasting results in most patients. The function and blood flow to the heart is improved following these procedures.

PHS-6040
(Rev. 10-76)

Project Description: 31 patients have undergone percutaneous transluminal coronary angioplasty (PTCA). All patients had symptoms of angina pectoris prior to the procedure; six patients were mildly symptomatic, 22 patients had moderate symptoms (angina with usual activities) and three patients had severe symptoms including symptoms at rest. Twenty three patients had single vessel coronary artery disease, seven had double vessel disease, and in one patient PTCA was attempted in a bypass vein graft. Nineteen (61%) patients had initial angiographic improvement in the coronary stenosis. In the remaining 12 patients the arterial stenosis could either not be crossed with the balloon catheter or was not compressable when the balloon was inflated to its maximum distending pressure. Of the 19 patients in whom there was initial angiographic improvement of the arterial stenosis, 14 improved and remained symptomatically stable up to 18 months after the procedure, and five developed recurrent severe symptoms with angiographically documented restenosis at the site of the original angioplasty. All cases of restenosis occurred within the first three months after PTCA. Of the stable patients, six have returned for coronary arteriograms six months after the initial PTCA, three have shown a reduction in the degree of stenosis compared to the immediate post-PTCA calculated stenosis (further improvement), and the other three remain angiographically stable.

To determine whether the improvement in the angiographic appearance of the coronary arteries would reduce exercise induced abnormalities in myocardial perfusion and function, perfusion (TL 201) and gated blood pool (Tc99m) scans at rest and exercise were performed prior to 10 to 21 days after, and subsequently six months following PTCA. Before PTCA 15 of the 19 patients who ultimately had a successful angiographic result had normal left ventricular blood pool and perfusion scans at rest. During exercise all patients developed angina and 14 of the 15 patients developed left ventricular wall motion abnormalities (ventricular tachycardia terminated exercise in the fifteenth patient). Left ventricular ejection fraction either remained unchanged or fell, and eight of the 15 developed perfusion abnormalities. PTCA reduced the average stenosis from 86% to 40% and the average gradient across the stenosis was reduced from 65 mm Hg to 28 mm Hg ($p<.01$). Following PTCA, none of these patients developed angina during exercise. Left ventricular function and perfusion were unchanged at rest. During exercise, only one of the 15 patients with prior wall motion abnormalities developed such abnormalities after PTCA. Ejection fraction during exercise increased in each of the patients from the rest value, and was greater than the value during exercise prior to PTCA in all except one. Average ejection fraction during exercise was 55% ± 3% pre-PTCA and increased to 64% ± 2% post-PTCA ($p<.05$). In patients who remained symptomatically stable, myocardial function at rest and during exercise was stable at the six months study following PTCA. The exercise induced perfusion abnormalities present in eight patients prior to PTCA were absent after PTCA. Thus, the results demonstrated that angiographic improvement obtained with PTCA leads to improved exercise capacity, LV function, and LV perfusion, all presumably reflecting augmentation of functionally important coronary blood flow.

The only complication of the procedure occurred in one patient, who developed an acute myocardial infarction 12 hours after PTCA. Myocardial infarction was uncomplicated and radionuclide angiogram prior to discharge showed a small new area of hypokinesia, but no change in the resting ejection fraction. Of the five patients who developed restenosis, three have undergone repeat angioplasty, two of whom improved and remain stable; the third developed restenosis after the second PTCA. This latter patient and the two other patients who initially developed restenosis have been operated upon.

We conclude PTCA can be performed in selected patients with coronary artery disease. Initial angiographic improvement can be obtained in over half the patients undergoing the procedure. At this point, the complication rate of the procedure appears to be low. Finally, when angiographic improvement occurs, it is accompanied by restoration of functionally important myocardial blood flow during exercise.

Publications: Kenneth M. Kent, M.D., Robert O. Bonow, M.D., Douglas R. Rosing, M.D., Lewis C. Lipson, M.D., Stephen L. Bacharach, Ph.D., Michael V. Green, M.S., Stephen E. Epstein, M.D.: Improved Myocardial Perfusion and Function Following Percutaneous Transluminal Coronary Angioplasty. Published in Proceedings of the Workshop on Percutaneous Transluminal Coronary Angioplasty, U.S. Department of HEW, PHS, NIH. June, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01764-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Unusual Locations of Hypertrophy 2-D Echo in Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	John S. Gottdiener	Head, Cardiology Consult.	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Wide angle two-dimensional echo may identify unusually located areas of cardiac hypertrophy in patients with a family history of hypertrophic cardiomyopathy, but normal M-mode echocardiogram and abnormal ECG. The diagnosis of hypertrophic cardiomyopathy, can thereby be established, despite a normal M-mode echo study.

PHS-6040
(Rev. 10-76)

Project description: M-mode echocardiography has proved useful in the diagnosis of hypertrophic cardiomyopathy with asymmetric septal hypertrophy. However, relatives of patients with hypertrophic cardiomyopathy may exhibit normal M-mode echos even though their ECG is abnormal. To determine whether such relatives have hypertrophic cardiomyopathy unrecognized by M-mode echo, 22 such patients were studied using wide-angle 2-dimensional (2-D) echo to reconstruct the geometry of the entire left ventricular wall. Patients were 5-49 years old (mean 17); 16 were asymptomatic. Most common ECG abnormalities were Q waves, ST-T changes and right ventricular hypertrophy. M-mode echos showed septal-free wall ratio <1.3 and no septal hypertrophy. In 17 (77%) of 22 patients, 2-D echo showed localized areas of left ventricular wall hypertrophy. Such "lumps" involved regions of the heart through which the M-mode beam does not usually pass - i.e., the posterior septum (7 patients), anterolaterlal left ventricular free wall (8 patients), and the ventricular septum near the apex (2 patients). Hence: 1) M-mode echo may not identify hypertrophic cardiomyopathy in certain patients; 2) in such patients ECG may be a more sensitive indicator of hypertrophic cardiomyopathy than M-mode echo; 3) wide-angle 2-D echo is necessary to identify these unusually located areas of cardiac hypertrophy.

Publications: Maron, B.J., Gottdiener, J.S., Bonow, R.O., Epstein, S.E.: Hypertrophic cardiomyopathy with unusual locations of left ventricular hypertrophy undetectably by M-mode echocardiography: Identification by wide-angle, two-dimensional echocardiography. Circulation, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01765-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Sudden Death in Young Athletes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	William C. Roberts	Chief, Pathology Branch	PB	NHLBI
	Hugh A. McAllister	Head, Cardiovascular Pathology. Armed Forces Institute of Pathology, Washington, D. C.		
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NIH, NHLBI
Armed Forces Institute of Pathology, Washington, D. C.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .45	PROFESSIONAL: .4	OTHER: .05

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Sudden death occurring in competitive athletes is usually due to structural congenital cardiovascular disease. The most common cause of such deaths in this series was hypertrophic cardiomyopathy. While the suspicion of cardiovascular disease is often raised during life in such athletes, the correct diagnosis is not often made clinically.

PHS-6040
(Rev. 10-76)

Project description: Youthful competitive athletes epitomize the conditioned, healthy segment of society. However, such athletes may die unexpectedly. To determine the causes of such sudden deaths, comprehensive necropsy studies were performed in 29 competitive athletes. Ages ranged from 14-30 years (mean 20). Sudden death occurred during or just after severe exertion in 26. The most common sports participated in were football and basketball. Structural cardiovascular alterations were identified in 28 of 29 patients, with the most common being hypertrophic cardiomyopathy (14 patients). Other diseases included idiopathic concentric left ventricular hypertrophy (5 patients), anomalous origin of the left coronary artery from anterior sinus of Valsalva (3 patients), coronary artery disease (3 patients), ruptured aorta with the Marfan syndrome (2 patients) and coronary artery hypoplasia (1 patient). Cardiac disease had been suspected clinically in 7 but in only one was the clinical diagnosis correct. Hence: 1) Sudden death in competitive athletes is usually due to structural cardiovascular disease; 2) the most common cause of sudden death was hypertrophic cardiomyopathy, while coronary artery disease was relatively uncommon. Knowledge of the causes of sudden death may serve as a basis of designing screening programs for prospective identification of cardiovascular disease in athletic populations.

Publications: Maron, B.J., Roberts, W.C., McAllister, H.A., Rosing, D.R., Epstein, S.E.: Sudden death in young athletes. Circulation 62:218-229, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01766-02 CB

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Specificity of Systolic Anterior Motion of the Anterior Mitral Leaflet (SAM)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB NHLBI
Other:	John S. Gottdiener	Head, Cardiology Consultation	CB NHLBI
	Lowell W. Perry	Staff Cardiologist	Children's Hosp. Med. Ctr.

COOPERATING UNITS (if any)

Department of Cardiology, Children's Hospital Medical Center, Washington, D. C.

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .11	PROFESSIONAL: .1	OTHER: .01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Systolic anterior motion of the anterior mitral leaflet (SAM) is not pathognomonic of hypertrophic cardiomyopathy, but is an uncommon finding in a large population of patients with a variety of other cardiac diseases. When SAM occurs it usually coexists with disproportionate septal thickening.

Project description: Systolic anterior motion of the anterior mitral leaflet (SAM) is a characteristic echocardiographic finding in patients with hypertrophic cardiomyopathy and left ventricular outflow obstruction. However, the value of SAM as a diagnostic marker for hypertrophic cardiomyopathy has been questioned, based on reported occurrences of SAM in other heart diseases. To determine the true specificity of SAM (under basal conditions) M-mode echocardiograms from 722 infants, children or adults with a variety of congenital or acquired heart diseases other than hypertrophic cardiomyopathy were reviewed. Ages ranged from 1 day to 75 years (median 40 years). True SAM was present in 22 (3%) of the 722 patients, yielding an overall specificity of 97%. SAM was particularly common in patients with transposition of the great vessels (11 of 52, 21%). When the study group was analyzed excluding patients with transposition of the great vessels, the prevalence of SAM was only 1.6%, giving a specificity of 98%. Of note, SAM was rarely found in patients with normal septal-free wall ratios (prevalence of 0.4%; specificity 99%). In conclusion: 1) SAM is an uncommon finding in a large population of patients with a variety of cardiac diseases other than hypertrophic cardiomyopathy; and 2) while SAM is not pathognomonic of hypertrophic cardiomyopathy, it is nevertheless a highly specific marker for that disease.

Publications: Maron, B.J., Gottdiener, J.S., Perry, L.W.: Specificity of systolic anterior motion of the anterior mitral leaflet (SAM) for hypertrophic cardiomyopathy: Prevalence of SAM in a large population of patients with other cardiac diseases. British Heart Journal, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01767-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Distribution of Hypertrophy by 2-Dimensional Echo in Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	John S. Gottdiener	Head, Cardiology Consultation	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .11	PROFESSIONAL: .1	OTHER: .01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In patients with hypertrophic cardiomyopathy, hypertrophy is asymmetric but often involves the anterolateral left ventricle and is not limited to the ventricular septum. Functional symptomatic limitation and outflow obstruction is most common in patients with marked, diffuse hypertrophy.

PHS-6040
(Rev. 10-76)

Project description: Wide-angle two-dimensional echocardiography was used to assess the pattern and distribution of left ventricular hypertrophy in 125 patients with hypertrophic cardiomyopathy. Hypertrophy involved substantial portions of both the ventricular septum and anterolateral left ventricular free wall in 54% of the patients, but was confined to the ventricular septum in 39%, and selectively involved only the left ventricular free wall in 7%. The posterior free wall, through which the M-mode beam passes, was usually the least hypertrophied region of the left ventricle.

Four patterns of distribution of left ventricular hypertrophy were identified in the study population. Compared with patients having other patterns of distribution, those patients with the most widespread hypertrophy involving most of the ventricular septum as well as portions of the left ventricular free wall: 1) more commonly experienced moderate to severe functional limitation (40 of 67 or 60% vs 14 of 58 or 24%; $p < 0.001$) and 2) more often demonstrated obstruction to left ventricular outflow under basal conditions (37 of 67 or 55% vs 10 of 58 or 17%; $p < 0.001$). Of note, hypertrophy was limited to the posterior or apical septum or anterolateral free wall in 18% of patients. In these patients conventional M-mode echocardiography failed to identify the presence of hypertrophy, and therefore the diagnosis of hypertrophic cardiomyopathy could be established only by two-dimensional echocardiography.

Hence, in patients with hypertrophic cardiomyopathy, wide-angle two-dimensional echocardiography is capable of detecting myocardial hypertrophy that involves a wide variety of patterns and is more extensive than may be appreciated by M-mode echocardiography. Although left ventricular hypertrophy is "asymmetric" in most patients with hypertrophic cardiomyopathy, it is not usually confined to the septum and often involves the anterolateral left ventricular free wall.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01768-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cellular Disorganization in the Left Ventricular Wall of Patients with Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Thomas J. Anan	Guest Worker	CB	NHLBI
	William Roberts	Head, Pathology Branch	PB	NHLBI

COOPERATING UNITS (if any)
Pathology Branch, NHLBI, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.11	.1	.01

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Using a quantitative method to assess the arrangement of cells in ventricular myocardium, we found that disorganized cardiac muscle cells are usually diffusely distributed throughout the septum and left ventricular free wall of patients with hypertrophic cardiomyopathy.

Project description: Hypertrophic cardiomyopathy is a disease of cardiac muscle in which premature sudden death often occurs. Marked disorganization of cardiac muscle cells in the ventricular septum is a highly specific and sensitive marker of hypertrophic cardiomyopathy. Extent and distribution of cellular disorganization in the left ventricular wall was assessed quantitatively in sections of ventricular septum and anterior and posterior left ventricular free wall from 52 patients with hypertrophic cardiomyopathy and 83 patients with other cardiac diseases, using substantial photographic enlargement and video planimetry. In patients with hypertrophic cardiomyopathy, ventricular septal disorganization was extensive (mean area disorganized 36 ± 4%). Disorganization was also marked in the left ventricular free wall (anterior and posterior), mean 26 ± 4%, and was as extensive in anterior left ventricular free wall (mean 32 ± 5%) as in ventricular septum. In control patients, mean area of left ventricular free wall disorganized was only 2%. The most marked degree of left ventricular free wall and combined ventricular septal and left ventricular free wall disorganization (mean 43 ± 6%) was present in 15 patients with hypertrophic cardiomyopathy ($\leq$25 years of age) in whom sudden death was the initial manifestation of heart disease. Hence: 1) in most patients with hypertrophic cardiomyopathy cellular disorganization is diffusely distributed in both ventricular septum and left ventricular free wall, and 2) this distribution of disorganization represents a diffuse cardiomyopathic process, particularly in young, previously asymptomatic patients who die suddenly.

Publications: Maron, B.J., Anan, T.J., Roberts, W.C.: Quantitative analysis of the distribution of cardiac muscle disorganization in the left ventricular wall of patients with hypertrophic cardiomyopathy. Circulation, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01770-02 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
NHLBI - Type II Coronary Intervention Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John F. Brensike	Coordinator	CB	NHLBI
Other:	M. Myrianthopoulos	Dietitian	CB	NHLBI
	Beverly Rogers	Registered Nurse	CB	NHLBI
	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI
	Robert Levy	Director	CB	NHLBI

COOPERATING UNITS (if any)
Cardiology and Lipid Metabolism Branches of the NHLBI

LAB/BRANCH
Cardiology Branch

SECTION
NHLBI - Type II Coronary Intervention Study

INSTITUTE AND LOCATION
NHLBI - NIH - Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	3	0

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A randomized, double blinded prospective study is currently in progress to determine whether cholesterol reduction will retard the progression of coronary artery disease, as assessed by serial angiographic studies at entry, and five years later. The study will be completed mid-1981.

PHS-6040
(Rev. 10-76)

NHLBI TYPE II CORONARY INTERVENTION STUDY

Project description: During the fiscal year 1980 the following things have happened:

1. The participants of the program are continuing to be processed in an orderly fashion and by October 1, 1980 over three-fourths of the Study participants will have concluded the Study.

2. No significant untoward side effects, no definitive evidence concerning medication efficacy has been reported.

3. Data transfer of all basic data to the Coordinating Center continues to be timely and complete.

4. Data entry and early analysis in the areas of family history, diet and exercise testing is beginning.

5. A paper titled "Reliability of assessing change in lesion diameter with sequential coronary angiography" has been written and is under consideration for publication in Circulation.

6. A Baseline Paper for the study is in preparation.

The next year should see continuing data collection of the remaining participants with the last participant receiving catheterization next summer. This will leave only analysis and reports to be done to conclude the program.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01772-01 CB

PERIOD COVERED

January, 1979 to May, 1980

TITLE OF PROJECT (80 characters or less)

Effects of Verapamil Alone and Combined with Propranolol on Left Ventricular Systolic Function in Patients with Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin B. Leon	Clinical Associate	CB	NHLBI
Other:	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec	NM	CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .25	PROFESSIONAL: .15	OTHER: .01

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Radionuclide cineangiograms were performed in 11 patients with coronary artery disease on various drug therapies. Verapamil caused modest diminution in left ventricular ejection fraction at rest, but no changes during exercise when compared with no medications. The combination of verapamil and propranolol caused no further worsening of left ventricular ejection fraction and was well tolerated.

Project description: Verapamil (V) appears to improve symptoms in pts with coronary disease (CAD), but controversy exists as to the magnitude of its negative inotropic effects and therefore its potential for causing cardiac failure. We therefore employed Tc99m radionuclide angiograms to assess the effects of LV systolic function at rest and during exercise (Ex) of V (480 mg/d), propranolol (P) (160-320 mg/d), and V+P in 11 pts with CAD, none of whom had rest ejection fraction (EF) <30%. V decreased rest EF (from 48±3 to 44±4%; p<.02) but did not change ejection rate (ER), measured in end-diastolic volumes/sec (EVD/s). P did not alter rest EF but decreased rest ER (from 2.5+.1 to 2.1±.1 EDV/s; p<.02). V+P decreased, from control, both rest EF (to 43±3%; p<.001) and rest ER (to 1.9+.1 EDV/s; p<.001). Rest EF decreased ≥5% in 5/11 pts (45%) on V and in 7/11 pts (64%) on V+P. However, no pt developed clinical CHF and the drugs did not change Ex EF. Thus, in pts with reasonable baseline LV function, V alone and with P causes mild to moderate depression of LV systolic function at rest, but not during Ex. This suggests V has the potential for deleterious effect but probably only in CAD pts with considerably reduced EF at rest.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01773-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of Verapamil on Left Ventricular Diastolic Filling in Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Martin B. Leon	Clinical Associate	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec	NM	CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.1	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To assess the effect of oral propranolol and oral verapamil on left ventricular systolic function and left ventricular diastolic filling in patients with coronary artery disease, we analyzed high temporal resolution time activity curves from radionuclide cineangiography at rest in 11 patients before and during therapy with each of these medications. Left ventricular ejection fraction was normal at rest in 8 of 11 patients, but diastolic filling was abnormal in all 11. Propranolol did not alter ejection fraction or diastolic filling. In contrast, verapamil significantly reduced ejection fraction, yet significantly improved diastolic filling, by increasing peak left ventricular filling rate and by reducing the time to peak filling rate. The improvement in diastolic filling, despite decreased ejection fraction, may account in part for the symptomatic improvement observed in many patients during verapamil therapy.

PHS-6040
(Rev. 10-76)

Project description: To determine the effects of propranolol and verapamil on left ventricular (LV) diastolic filling in patients with coronary artery disese (CAD), high resolution (10-20 msec/frame) time-activity curves from resting gated Tc99m radionuclide angiograms were analyzed in 11 symptomatic patients before (C) and during oral propranolol (160-320 mg/day) and oral verapamil (480 mg/day). LV ejection fraction (EF) was normal at rest in 8 of 11 patients, but abnormal LV diastolic filling, defined as peak filling rate (PFR) <2.5 end-diastolic volumes (EDV)/sec or time to PFT >180 msec, was present in all patients. Propranolol resulted in decreased heart rate (HR), decreased peak ejection rate (PER) (C=2.5±.1, p=2.1±.1 EDV/sec, p<.01), and increased ejection time (ET) (C=319±11, P=371±9 msec, p<.005), but did not change EF, PFR or time to PFR. During verapamil, despite decreased HR and EF (C=48±3, V=44±4%,p<.05), PFR increased (C=1.9±.2, V=2.3±.3 EDV/sec, p<.05), and time to PFR decreased (C=190±13, V=161±8 msec, p<.05), while PER was unaffected. Both PFR and time to PFR improved in all patients during verapamil except the 2 patients with the greatest decrease in both EF and HR. The improvement in LV diastolic filling, despite decreased EF, may account in part for the symptomatic improvement observed in many patients during verapamil therapy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01774-01 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Left Ventricular Systolic Function and Diastolic Filling in Patients with Hypertrophic Cardiomyopathy: Effect of Verapamil

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Head, Applied Physic Sec.	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diag.	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	John R. Condit	Research Assistant	CB	NHLBI
	Martin B. Leon	Clinical Associate	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Cardiovascular Diagnosis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To assess mechanisms responsible for the clinical improvement observed in patients with hypertrophic cardiomyopathy while on verapamil therapy, we examined high temporal resolution time activity curves obtained from radionuclide cineangiography at rest in 45 patients before and after oral verapamil administration. Sixteen patients were also studied during oral propranolol therapy. Left ventricular systolic function was normal or supra-normal in all patients, but left ventricular diastolic filling was subnormal in 70% of patients. Systolic function was not altered during verapamil, but peak left ventricular filling rate significantly increased and time to peak filling rate significantly shortened. In contrast, propranolol did not alter diastolic events, but prolonged left ventricular ejection time and reduced ejection rate. Thus, verapamil improves abnormalities in left ventricular diastolic filling without altering systolic function. This mechanism may contribute to the clinical improvement of many patients with hypertrophic cardiomyopathy during verapamil therapy.

PHS-6040
(Rev. 10-76)

Project description: Verapamil (V) improves exercise capacity in patients with hypertorphic cardiomyopathy (HCM), but its mechanisms of action are unknown. We therefore examined the effect of oral verapamil (320-640 mg/day) resting left ventricular (LV) systolic and diastolic function in patients with HCM. High resolution time activity curves from gated Tc99m radionuclide angiograms were analyzed before (C) and after verapamil therapy in 45 patients, of whom 16 were also studied during propranolol therapy. Systolic function was normal or supra-normal in all patients. However, diastolic dysfunction was evident in 70%. Verapamil did not change LV ejection fraction (EF), peak ejection rate (PER) or ejection time (ET). However, verapamil increased peak LV filling rate (FR) (C=3.3±0.1 [±SD], V=4.1±0.1 end-diastolic volumes (EDV)/sec, $p<0.001$) and reduced time to peak FR (C=181±48,V=157±34 msec, $p<0.001$). In contrast, propranolol did not change EF, FR, or time to peak FR but did prolong ET (C=364±48, P=400±48 msec;$p<0.001$) and reduce PER (C=3.7±.7, P=3.4±.6 EDV/sec, $p<0.001$. Thus, 1) LV diastolic filling is abnormal in a high % of patients with HCM; 2) verapamil normalizes or improves these abnormalities without altering systolic function. This mechanism may contribute to the clinical improvement of many HCM patients during verapamil therapy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01775-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Left Ventricular Diastolic Filling in Patients with Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Martin B. Leon	Clinical Associate	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.4	.2	.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To evaluate left ventricular diastolic filling in patients with coronary artery disease, we analyzed high resolution time activity curves obtained from gated Tc99m radionuclide cineangiography at rest in 231 patients. Peak left ventricular filling rate (normal $\geq$2.5 end-diastolic volume/sec) or time to peak filling rate (normal $\leq$180 msec) was abnormal in 90% of all patients with coronary artery disease, 86% of patients with normal left ventricular ejection fraction, 83% with normal ejection fraction without Q waves on ECG. Thus, left ventricular diastolic filling was abnormal in most patients with coronary artery disease independent of left ventricular systolic function, even in patients without evidence of prior myocardial infarction.

Project description: To assess left ventricular (LV) diastolic filling at rest in patients with coronary artery disease (CAD), we analyzed high resolution time activity curves (10-20 msec/frame) obtained from gated Tc99m radionuclide angiograms in 231 patients. Peak LV filling rate (PFR), in end-diastolic volumes (EDV)/sec, was subnormal in CAD patients (1.8±0.6 [±SD] vs normal 3/3±0.6, p<0.001) and time to PFR (TPFR), measured from end systole to PFR, was prolonged (171±41 msec vs normal 133±22 msec, p<0.001). These indices were also abnormal in the 141 patients with normal resting LV ejection fraction (EF) (PFR=2.1±0.5 EDV/sec; TPFR=175±36 msec) and in 1234 patients without Q waves on ECG (PFR=2.1±0.5 EDV/sec; TPFR=168±38 msec). Abnormal LV filling at rest (PFR ≤2.5 EDV/sec or TPFR>180 msec) was found in 90% of all patients with CAD, 86% of patients with normal rest LVEF, and 85% of patients without Q waves, values comparable to the sensitivity achieved by analysis of LVEF response to exercise in CAD patients. When analysis of diastolic filling, exercise LVEF and wall motion were combined, sensitivity in detecting CAD was 99%. Thus, 1) diastolic filling is abnormal in most patients with CAD at rest independent of LV systolic function; 2) radionuclide assessment of systolic and diastolic function can identify almost all patients with CAD.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01776-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Predictive Value of Exercise Capacity and Ejection Fraction Response to Exercise in Mildly Symptomatic Patients with Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec	NM	CC
	Carolyn J. Ewels	Research Assistant	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH

Cardiology Branch, Bethesda, Maryland 20205

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.1	.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We performed radionuclide cineangiography at rest and during exercise in 120 patients with coronary artery disease who were not considered candidates for coronary artery bypass surgery because of mild symptoms. Rest or exercise left ventricular ejection fraction were not different between the 99 patients who have remained stable (mean follow-up two years), the 15 patients who have had increased angina requiring operation, and the six patients who have died suddenly. However, the change in ejection fraction from rest to exercise, and the exercise load achieved, identified subgroups with different 2 year event rates. Patients with a decrease in ejection fraction with exercise had significantly worse prognosis than those with an increase in ejection fraction. Among patients with a decrease in ejection fraction during exercise, patients who achieved ≤80 watts work load had a worse prognosis than patients with better exercise tolerance. Thus, the change in ejection fraction from rest to exercise as well as exercise capacity helps to define subgroups at high and at low risk.

Project description: To determine if left ventricular ejection fraction (EF) response to exercise, a measure of reversible ischemia, identified patients with coronary artery disease (CAD) who are at risk of sudden death (SD), 120 patients with CAD who were not considered candidates for coronary artery bypass surgery (CABG) because of mild symptoms were studied by gated Tc99m radionuclide angiography. Rest or exercise EF was not different among patients who have remained stable (mean follow-up 2 yrs), patients who have developed increased angina (A) requiring CABG, or patients who have had SD. However, change (Δ) in EF from rest to exercise, and exercise load achieved (EX) identified subgroups with different 2 year event rates (*=p<.01):

	EX<80 watts			EX>80 watts		
ΔEF	SD	A	Total	Total	SD	A
↑	0/5	0/5	0/5	2/36	0/36	2/36
*				*		
↓	3/25	7/25	10/25	9/54	3/54	6/54

Thus, 1) the level of EF at rest or during exercise does not predict which CAD patients with mild symptoms will die or progress, but 2) the change in EF from rest to exercise as well as exercise capacity helps to define a group at high and at low risk.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01777-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Microsphere "loss" from noninfarcted myocardium: implications for measurement of blood flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Nancy J. Davenport	Staff Fellow	CB	NHLBI
Other:	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Roberto Bolli	Visiting Fellow	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER: 0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Radioactively labelled microspheres are commonly used to measure coronary blood flow. Decrease in microsphere content is known to occur in infarcted myocardium, introducing a potential source of error in flow measurement. To investigate stability of microsphere content in ischemic but noninfarcted muscle, we injected microspheres in 11 open-chest dogs and produced coronary occlusion. Four hours later microsphere content of ischemic endocardium fell to 78% of the value measured in normal endocardium. Such changes were not seen in ischemic epicardium. An additional 14 closed-chest dogs had preocclusion microspheres and sacrifice 3 days later. In these animals, microsphere content was decreased (to 55-67% of analogous normal zone values) approximately equally in ischemic but surviving muscle and in infarcted muscle. We conclude that microsphere "loss" may distort flow values even in relatively short experiments. Within 3 days microsphere "loss" occurs in both surviving and infarcting portions of myocardium in the region supplied by the occluded artery. This uniformity permits comparison of flow among various subdivisions of ischemic myocardium.

Project description: Preocclusion microsphere (M) content decreases in ischemic myocardium 24 hours after coronary occlusion (CO). This M loss, a potential source of error in blood flow (BF) measurement, has been attributed to dissolution of infarcting tissue. However, we have also observed M loss from ischemic myocardium that is not infarcted. We evaluated persistance of 15u M, injected prior to CO, in nonischemic (N) region and risk region (RR) (i.e., region distal to occluded artery) 4 hrs after open-chest left anterior descending artery (LAD) CO in 11 dogs. Ischemia was documented by low post-CO flows in the RR. Pre-CO content in RR endocardium (endo) was 21% lower than N endo, indicating M loss; similar evidence of M loss was not seen in RR epicardium (epi) although both RR epi and RR endo had a 4% increase in water content. To determineeeeeeen of M loss within RR we injected 15u M prior to closed-chest LAD CO in a second group of 24 dogs. Three days later, RR of the left ventricle was determined by post-mortem dye perfusion and I identified by triphenyltetrazolium chloride. RR was divided into infarct (I), medial regions adjacent to I (MA) and remote from I (MR) and lateral regions adjacent to I (LA) and remote from I (LR). N epi BF was .93 ml/min/g and N endo .99. Each subdivision of the RR had significant M loss as compared to N epi and N endo ($p>.01$). Mean M losses were: I, 38%; MA, 45%; MR, 36%; LA, 38%; LR, 33%. M loss did not differ significantly among the subdivisions of the RR. Thus in acute ischemia: 1) M loss occurs in ischemic endo by 4 hr after CO; 2) within 3 days M loss occurs uniformly in both infarcted and surviving portions of the RR. This uniformity permits use of M to compare BF among divisions of the RR; 3) dissolution of infarcting tissue does not appear solely responsible for M loss.

Publications: Davenport NJ, Goldstein RE and Bolli R: Microsphere "loss" from noninfarcted myocardium: implications for measurement of coronary blood flow. Clin Res 28:164A, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01778-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Blood Flow to Surviving and Infarcting Myocardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert E. Goldstein	Senior Investigator	CB	NHLBI
Other:	Nancy J. Davenport	Staff Fellow	CB	NHLBI
	Roberto Bolli	Visiting Fellow	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Section on Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

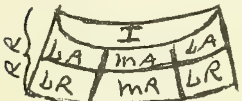
☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

After coronary occlusion myocardium originally supplied by the occluded artery ultimately separates into infarct and surviving muscle. To clarify circumstances leading to infarction, we compared the time course of collateral blood flow to muscle that later became infarct and collateral flow to muscle that survived despite inclusion within the distribution of the occluded artery. Permanent left anterior descending occlusion was produced in 14 dogs. Microspheres were given to measure blood flow prior to occlusion and 5 min, 20 min and 4 hr after occlusion. Dogs were sacrificed 3 days later and their hearts stained to identify the various tissue regions. Although blood flow was reduced to all regions supplied via collaterals, each subdivision of surviving muscle had higher flow than infarcting muscle as early as 5 min after coronary occlusion. Blood flow characteristics of each muscle type identify the fate of tissue very early after coronary occlusion. Blood flow increases as distance from infarcting muscle increases, with greater increase in tissue lying at the lateral borders of the region supplied by the occluded artery.

PHS-6040
(Rev. 10-76)

Project description: After coronary occlusion (CO) myocardium originally supplied by the occluded artery (=region at risk or RR) ultimately separates into infarct (I) and surviving muscle (SM). To clarify circumstances leading to I we compared the time course of collateral blood flow (BF) to I and to surviving portions of the RR. Permanent left anterior descending CO was produced in 14 closed chest dogs. Microspheres (15) were given prior to CO and 5 min, 20 min and 4 hr after CO. Three days later dogs were sacrificed, RR identified by Evans blue and fluorescein perfusion of the intact heart, and I demonstrated in horizontal slices stained with triphenyltetrazolium chloride. RR was divided into I and SM. SM was subdivided into lateral regions adjacent to I (LA) and remote from I (LR), and medial regions adjacent to I (MA) and remote from I (MR), to assess anatomical differences in BF distribution.



Five min after CO BF (ml/min/g) was .99±.09 to normally perfused (non-RR) endocardium and .93±.09 to normal epicardium. RR BF was as follows (Mean ± SE):

epicardium	I	MA	LA	MR	LR
5 min flow(ml/min/g)	.02±.01	.08±.03	.22±.05	.19±.04	.27±.05
20 min flow	.04±.01	.13±.02	.24±.03	.34±.05	.45±.07
4 hr flow	.08±.03	.24±.05	.33±.06	.48±.02	.57±.12

Although BF was reduced throughout RR, each subdivision of SM had higher BF than I as early as 5 min after CO and had a more rapid rise of absolute BF within 4 hr after CO. Thus, BF characteristics of I and SM identify the fate of tissue very early after CO. BF within RR increases as distance from I increases, with lateral segments increasing more than medial segments.

Publications: Goldstein RE, Davenport NJ, Bolli R and Epstein, SE: Blood Flow to Surviving and Infarcting Myocardium. Clin. Res. 28: 174A, 1980

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01779-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effects of _in_ _vivo_ and _in_ _vitro_ verapamil on _in_ _vitro_ platelet aggregation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. J. Davenport	Staff Fellow	CB	NHLBI
Other:	R. E. Goldstein	Senior Investigator	CB	NHLBI
	D. R. Rosing	Senior Investigator	CB	NHLBI
	M. Leon	Clinical Associate	CB	NHLBI
	J. Jordan	Clinical Associate	CHB	NIAMDD
	N. R. Shulman	Chief, Clin. Hematology Branch	CHB	NIAMDD

COOPERATING UNITS (if any)

Clinical Hematology Branch, NIAMDD, NIH

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.5	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil, an investigational drug useful in cardiovascular disease, is known to inhibit calcium movement across cell membranes. _Platelet aggregation_, potentially important in cardiovascular disease, is dependent upon cellular calcium transport. Therefore, patients receiving verapamil might also experience an alteration in platelet aggregability. Over a wide range of drug blood levels, patients receiving verapamil did not show any alterations in platelet response. However, when verapamil was added to platelet in vitro at 20-40x the clinical blood levels, platelet aggregation was inhibited.

PHS-6040
(Rev. 10-76)

Project description: Verapamil (V) has been suggested as a potential inhibitor of platelet aggregation due to its interference with calcium transport across plasma membranes. To determine effects of therapeutic doses, we examined platelet aggregation in 13 patients with hypertrophic cardiomyopathy before and during V therapy. Platelet aggregation was measured as change in light transmission through standardized platelet rich plasma after ADP or epinephrine. Mean changes (expressed as percent of difference in light transmission between platelet poor plasma and platelet rich plasma) indicated a lack of V-induced inhibition:

	ADP			Epinephrine		
	2x10 M	2x10 M	2x10 M	10 M	10 M	10 M
Pre V	86±2	74±3	42±7	83±4	86±2	83±3
On V	83±3	74±3	48±6	79±7	82±6	76±5

V plasma level (mean 184±36 range 59-541 ng/ml) did not correlate with aggregation response. *In vitro* addition of V, 25 M (10,000 ng/ml), to platelets of 6 normal subjects inhibited aggregation to epinephrine (10 M). ADP induced aggregation (2x10 M) was inhibited by V, 50 M, whereas aggregation induced by the calcium ionophore A23187 at 10 M was not. Our data show that V is capable of inhibiting platelet aggregation. However, inhibition is not seen within the therapeutic range in patients with normal platelet function.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01780-01 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Irreversibility of Sudden Death Post-Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Beverly Jones Collins	Visiting Fellow	CB	NHLBI
Other:	Randolph Patterson	Senior Investigator	CB	NHLBI
	Roger Aamodt	Chief, Whole Body Counter Section	NM	CC

COOPERATING UNITS (if any)

Whole Body Counter Section, NM, CC

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.3	.2	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We analyzed a series of dogs in whom heart attacks were produced under observation. All who exhibited severe heart rhythm disturbances died despite optimum treatment. It appears death due to rhythm abnormalities after a heart attack may not be avoidable despite observation and early treatment.

PHS-6040
(Rev. 10-76)

Project description: Sudden death (SD) is believed reversible if resuscitation is begun immediately. A logical assumption relates unsuccessful resuscitation to a larger % of left ventricle supplied by the occluded arteries (RR) and a lower collateral blood flow (CBF) to the ischemic zone (IZ). We retrospectively analyzed data from 45 conscious dogs who underwent closed chest coronary artery occlusion (CO) during continuous monitoring of left atrial and aortic pressures and ECG. Microsphere measurements of CBF were made intermittently. 28 dogs experienced SD under observation (ventricular fibrillation <5 hrs. post-CO) and an attempt at resuscitation failed in each case despite prompt cardioversion, ventilation, appropriate cardiac medications and right ventricular pacing. RR was measured by postmortem staining. Surprisingly, there was no statistically significant difference between the SD and survivor groups' transmural mean (.13 vs .17 ml/min/g, n=12 vs n=8), end-(.04 vs .06 ml/min/g), mid- or epicardial CBF to the IZ 5 min post-CO. Also, RR was the same in both groups (.49 n=8 and .48 n=17). We conclude that SD in this experimental model may be irreversible despite optimal resuscitation and this irreversibility is not explainable by RR size or changes in CBF.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01781-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cardiac Electrical Instability after Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Beverly Jones Collins	Visiting Fellow	CB	NHLBI
Other:	Randolph Patterson	Senior Investigator	CB	NHLBI
	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI
	Roger Aamodt	Chief, Whole Body Counter Section	NM	CC

COOPERATING UNITS (if any)

Whole Body Counter Section, NM, CC

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .6	PROFESSIONAL: .3	OTHER: .3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We found that ease of provocation of rhythmn disturbances in the dog heart 3-4 days after a heart attack correlates very well with heart attack size. Diminishing heart attack size may therefore decrease the risk of rhythm disturbance in this situation.

We also made observations about the type and placement of stimulation necessary to produce abnormal cardiac rhythms in these dogs. This information may facilitate the testing of electrical instability in other settings.

Project description: No direct evidence exists relating size of acute myocardial infarction (AMI) to electrical instability (EI) several days later. Moreover, although it has been suggested that an increase in salvaged myocardium may increase EI post AMI, direct data are lacking. We therefore studied 12 conscious dogs 3 to 4 days after AMI was produced by closed chest coronary artery occlusion. The right ventricle (RV) was stimulated by a series of single, followed by double stimuli, scanning from end-diastole toward the T wave during continuous RV pacing. Abnormal responses included: repetitive ventricular responses, short runs of ventricular tachycardia (VT), sustained VT and ventricular fibrillation. A score of EI was determined by the type of responses, the nature of the stimuli required to produce the responses (i.e., single or double) and the time in diastole at which they could be elicited. EI correlated directly with MI size (r=.96). The percent of the risk region (RR) and left ventricle (LV) naturally salvaged (RR-MI/RR or LV) were inversely correlated with EI (r=.94, -.75). This was due mainly to the inverse relation between % LV infarcted and % LV salvaged (r=.87). We conclude that 1) EI determined by electrical stimulation several days post-AMI increases in proportion to side of MI and 2) LV salvaged by intervention may enhance electrical stability post-MI.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01782-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Difference in Collateral Blood Flow After Abrupt vs. Gradual Coronary Occlusions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Randolph E. Patterson	Senior Investigator	CB	NHLBI
Other:	Beverly A. Jones Collins	Visiting Fellow	CB	NHLBI
	Roger Aamodt	Chief, Whole Body Counter Section	NM	CC

COOPERATING UNITS (if any)

Whole Body Counter Section, Dept. of Nuclear Medicine

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/8	1/4	1/8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The rate of coronary occlusion during acute myocardial infarction in man must vary widely, and it is not known how this vartiation may influence the severity of ischemic injury. This animal study demonstrates a 30% greater increase in coronary collateral blood flow when total occlusion was preceded by a 45 min gradual progressive stenosis rather than abrupt occlusion. Thus, interventions which can slow the rate of progression of coronary occlusion may reduce the severity of resulting myocardial ischemic injury by allowing greater collateral flow.

Project description: While rate of coronary occlusion (CO) in human myocardial infarction (MI) may vary widely, it is not known whether collateral myocardial blood flow (MBF) after total CO varies with the rate of stenosis. In 16 open chest dogs we measured aortic pressure (AP), coronary blood flow (CBF) by flowmeter, and collateral MBF by microspheres. Each of 8 Group I dogs had both abrupt and gradual (over 45 min) CO in random sequence; we measured MBF 1.5 min after total CO was achieved. Each of 8 Group II dogs had MBF measured 1.5 and 45 min after abrupt CO. Transmural mean (TM) and subendocardial (endo) collateral MBF 1.5 min after total CO in Group I was 30% higher after gradual CO (TM=0.18, endo-0.8 ml/min/g compared to abrupt CO (TM=0, 14, endo=0.06 ml/min/g, $p<0.05$). In contrast, 45 min after abrupt total CO in Group II, collateral MBF had not increased from its 1.5 min value. We conclude that collateral MBF to TM and endo are higher after total CO if it is preceded by 45 min gradual CO. This suggests that agents which slow the rate of CO (e.g., by spasm or platelet aggregation) may enhance collateral MBF and reduce ischemic injury. Also, the failure of 45 min sustained total CO to stimulate increased collateral flow suggests inhibition of collateral development (relative to gradual CO) by unknown mechanisms related to more severe ischemia (sustained total CO).

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01783-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Sensitivity and Specificity of Radionuclide Angiography in Coronary Artery Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert O. Bonow	Senior Investigator	CB	NHLBI
Other:	Stephen L. Bacharach	Physicist	NM	CC
	Michael V. Green	Chief, Applied Physics Sec	NM	CC
	Kenneth M. Kent	Head, Cardiovascular Diagnosis	CB	NHLBI
	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Lewis C. Lipson	Senior Investigator	CB	NHLBI
	Jeffrey S. Borer	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

Nuclear Medicine Department, CC, NIH

LAB/BRANCH

Cardiology Branch

SECTION

Cardiovascular Diagnosis

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .3	PROFESSIONAL: .2	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To determine the sensitivity of radionuclide cineangiography in detecting coronary artery disease in patients without previous myocardial infarction, we evaluated 231 patients with coronary artery disease. Regional and global left ventricular function at rest and during exercise were assessed by analysis of regional wall motion and determination of left ventricular ejection fraction. Ninety-nine of the 231 patients had normal left ventricular wall motion, normal left ventricular ejection fraction at rest, and no Q wave on ECG. Abnormalities in regional or global left ventricular function were observed in 89 of these patients (89%). Specificity of the technique was determined in 49 patients with chest pain and normal coronary arteries; specificity was 94%. Thus, radionuclide cineangiography is sensitive and specific in detecting coronary artery disease even in patients with normal left ventricular ejection fraction at rest and without prior myocardial infarction.

PHS-6040
(Rev. 10-76)

Project description: Gated Tc99m radionuclide angiography at rest and during exercise is highly sensitive in detecting coronary artery disease (CAD). Many patients with CAD, however, have previous myocardial infarction (MI) with abnormal left ventricular (LV) ejection fraction (EF) at rest or Q waves on EKG. Inclusion of such patients in studies of LV function will enhance sensitivity figures. To assess the sensitivity of radionuclide angiography in detecting CAD in patients without prior MI, we studied 231 patients with CAD. Specificity was assessed in 49 patients with chest pain and normal (nl) physical exam, EKG, echocardiograms, and coronary arteriograms. We found the following sensitivity of rest and exercise LVEF and wall motion abnormalities (WMA) for detecting CAD:

	#	LVEF	WMA	LVEF+WMA
1) All patients	231	90%	93%	94%
2) Nl Rest LVED	141	85%	89%	90%
3) No Q Waves	123	88%	90%	91%
4) Nl Rest LVEF, No Q Waves	110	86%	89%	90%
5) Nl Rest LVEF, No Rest WMA, and No Q Waves	99	86%	88%	89%
Specificity	49	94%	98%	94%

Thus, radionuclide angiography is sensitive and specific in detecting CAD even in patients with normal rest LVEF and without prior MI.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01784-01 CB

PERIOD COVERED
October 1, 1979 to September 30 ,1980

TITLE OF PROJECT (80 characters or less)
Collateral Flow Reserve in Surviving Subepicardium After Non-Transmural Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Randolph E. Patterson	Senior Investigator	CB	NHLBI
Other:	Beverly A. Jones Collins	Visiting Fellow	CB	NHLBI
	Roger Aamodt	Chief, Whole Body Counter Section	NM	CC
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI
	John Bacher	Chief, Surgical Section Veterinary Resources Br.	VRB	DRS

COOPERATING UNITS (if any)
Whole Body Counter Section, Dept. of Nuclear Medicine, Clinical Center Surgical Section, Veterinary Resources Branch, Division of Research

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/4	1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

After non-transmural myocardial infarction, the coronary collateral blood flow to non-infarcted subepicardial tissue returns toward normal in the resting state. We studied dogs 3-4 days after non-transmural MI and found that the collateral flow to surviving subepicardium was 56% of normal zone subepicardium at rest, but only 35% of normal zone during stress induced by pacing tachycardia. These data suggest the potential for stress to induce angina or other ischemic complications in surviving myocardium within the region of supply of an occluded coronary artery.

Project description: During non-transmural myocardial infarction (MI) subendocardium (endo) within the distribution of an occluded coronary artery infarcts, and sub-epicardium (epi) is spared. While collateral myocardial blood flow (MBF) to surviving epi returns toward normal at rest, its capacity to respond to stress is unknown. Thus, we induced MI in 9 awake dogs; 3-4 days later we measured 1) pressures in aorta (AP) and left atrium (LAP) and 2) microsphere MBF during the control state (CS) and pacing-tachycardia (PT). Myocardium supplied by the occluded artery (ischemic zone, IZ) and infarct size (MI) were measured by post-mortem staining: MI/IZ was 39±25%. Results represent MBF (ml/min/g) to normal (NZ) or IZ: *=p<.05, CS vs PT; and each IZ value is lower than corresponding NZ, (p<.05).

	HR	AP	LAP	EN-NZ	EN-IZ	EPI-NZ	EPI-IZ
CS	113	103	10	1.55	.23	1.39	.78
PT	239	107	13	2.58*	.06*	2.89*	1.01

Thus, although resting collateral MBF to surviving epi doubled between 1 hr and 3-4 days after MI, the capacity for MBF to increase during stress is markedly compromised. These data demonstrate the potential for stress to induce angina, arrhythmic death, or extension of the MI across its transmural border zone.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01785-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies with Antibodies to Myosin Kinase Isolated from Smooth Muscle

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Primal deLanerolle	Staff Fellow	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	William Anderson	Chemist	CB	NHLBI
	J. Maurice Miles	Medical Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)

Dr. James Feramisco)
Dr. Keith Burridge)-- Cold Spring Harbor Labs., Cold Spring Harbor, N. Y.

LAB/BRANCH

Cardiology Branch

SECTION

Molecular Cardiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.6	1.2	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies to the enzyme myosin light chain kinase have been purified and will be used to study the role of this enzyme in regulating smooth muscle contraction as well as contractile activity in non-muscle cells such as human platelets.

199

Project description: We have been studying the role of myosin phosphorylation in regulating contraction. In order to do so, we have purified myosin light chain kinase from turkey gizzard smooth muscle and developed an antibody to the purified kinase in rabbits. The kinase antibody has been purified from rabbit serum by affinity chromatography. This purified antibody has been shown to inhibit the catlytic activity of turkey gizzard myosin light chain kinase. Pre-immune serum and antiserum treated with purified kinase have no effect on kinase activity.

We have used this antibody, in collaboration with Dr. James Feramisco of Cold Springs Harbor Laboratory, to determine the intracellular localization of myosin light chain kinase in non-muscle cells. Data from initial experiments indicate that myosin light chain kinase is localized within fibroblasts on filamentous structures known to contain actin and myosin. Studies are presently being conducted to (1) determine the distribution of myosin light chain kinase within other cell types and (2) to determine the antigenic similarities between kinases from various tissues.

Data from these experiments should provide additional information on the importance of myosin phosphorylation in regulating contraction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01786-01 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James R. Sellers, Jr.	Guest Worker	CB	NHLBI
Other:	Robert S. Adelstein	Head, Molecular Cardiology	CB	NHLBI
	William Anderson	Chemist	CB	NHLBI
	J. Maurice Miles	Medical Biology Technician	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Section on Molecular Cardiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.2	OTHER: 0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanism of phosphorylation-dependent myosin-linked regulation is being investigated using turkey gizzard smooth muscle and Limulus striated muscle myosins as model systems. Enzymatically active subfragments of the myosins will be prepared to allow a kinetic analysis of this mechanism.

201

Project description: It was discovered that myosin from the striated muscle of *Limulus*, the horseshoe crab, like smooth muscle myosin is regulated by a calcium dependent phosphorylation. Myosin kinase from *Limulus* has been partially purified. The site of phosphorylation is the regulatory light chain in *Limulus* myosin and in smooth muscle myosin. The MgATPase activity of the unphosphorylated *Limulus* myosin is not activated by actin whereas that of the phosphorylated myosin is. A reconstituted system of gizzard kinase, calmodulin, rabbit actin, rabbit tropomyosin, and *Limulus* myosin has a calcium sensitive MgATPase activity. This calcium sensitivity is correlated with the calcium dependent phosphorylation of the light chain. The phosphorylated myosin's actin activated MgATPase is high even in the absence of calcium ions.

This is further evidence that *Limulus* myosin behaves similarly to vertebrate smooth muscle myosin and represents an excellent model system. Its advantages over smooth muscle myosin are three fold: 1) *Limulus* myosin can easily be obtained relatively free of kinase and phosphatase; 2) it is less labile; 3) it has about an eight fold higher actin activated MgATPase than smooth muscle myosin.

Current studies underway include plans to purify the *Limulus* myosin kinase and phosphatase, preparation of enzymatically active subunits of *Limulus* and gizzard myosin, and analysis of the kinetics of the phosphorylation dependent regulation using these subunits. Preliminary work indicates that all three of these proposed studies are feasible.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01787-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of Ischemia on Myocardial Proteolysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roberto Bolli	Visiting Fellow	CB	NHLBI
Other:	Nancy J. Davenport	Staff Fellow	CB	NHLBI
	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Section on Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL: .5	OTHER: .5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tyrosine production (an index of the overall rate of cellular proteolysis) was measured in slices of 1) normal canine myocardium, 2) 1-hour ischemic endocardium, 3) 3-hour ischemic epicardium, and 4) 3-hour ischemic endocardium each incubated in "aerobic" or "ischemic" medium. Tyrosine production of normal myocardium in "aerobic" medium was greater than that of ischemic tissues in "ischemic" medium, and was similar to that of ischemic tissues in "aerobic" medium. Thus, cellular proteolysis appears decreased during acute myocardial ischemia, suggesting that cellular proteases do not contribute to ischemic cell death.

Project description: Several studies suggest that during acute myocardial ischemia (AMI) lysosomal hydrolases contribute to the death of cells that otherwise may have remained viable and eventually regained function. However, the effect of AMI, produced in intact animals, on proteolysis (P) has not been evaluated. Thus, we ligated the left anterior descending coronary artery in 29 open-chest dogs; .5 mm thick slices were obtained from 1-hr ischemic endocardium (1-EN), 3-hrs ischemic endocardium (3-EN) and epicardium (3-EP) and from control myocardium (C). Slices were incubated for 1, 2 or 3 hrs in "aerobic" medium (Krebs-Ringer-bicarbonate {KRB} buffer, with glucose 10mM and insulin .1 U/ml, saturated with 95% O_2, - 5% CO_2, pH 7.4) or in "ischemnic" medium (KRB saturated with 90% N_2 - 10% CO_2, pH 6.8). The protein synthesis inhibitor cycloheximide (CH) was added to both media (.5mM) to block amino acid reutilization. CH did not inhibit P. The overall rate of P was measured by net production of tyrosine by the slices (pmol/mg of tissue, mean ± SE):

	"aerobic" medium				"ischemic " medium			
Incubation	C	3-EP	I-EN	3-EN	C	3-EP	I-EN	3-EN
1 hour	25±2	22±2	20±4	20±2	17±2	12±I	15±3	19±2
2 hours	47±4	-	38±5	-	22±6	-	24±3	27±3
3 hours	72±4	58±4*	54±6	50±6	38±4	25±I*	34±4	27±3*

(* = $p<.05$ vs C). A similar pattern was obtained when the production of tyrosine was related to the concentration of protein in the slices. We conclude that P does not increase in AMI either before irreversible damage is believed to occur (3-EP) or shortly after (1-EN, 3-EN). This suggests that cellular proteases do not cause cell death during AMI, making it unlikely that drug-induced inhibition of P would be an important mechanism responsible for preventing ischemic myocardium from becoming necrotic.

Publications: Bolli, R., Davenport, N.J., Goldstein, R.E., Epstein, S.E.: Effect of Ischemia on Myocardial Proteolysis. Am J Cardiol 45: 414, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01788-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Cellular Proteases in the Development of Acute Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roberto Bolli	Visiting Fellow	CB	NHLBI
Other:	Richard O. Cannon	Clinical Associate	CB	NHLBI
	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Section on Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In vivo pretreatment with leupeptin (an inhibitor of thiol-proteases) markedly reduced cellular proteolysis (measured as tyrosine production) in slices of normal (-42%) and ischemic (-73%) rat ventricular myocardium incubated in vitro. However, the same doses of leupeptin failed to reduce infarct size (determined histologically) in rats. Thus, thiol-proteases (lysosomal cathepsins B, H and L and cytoplasmic (Ca-activated protease) do not appear to contribute to ischemic cell death.

PHS-6040
(Rev. 10-76)

Project description: Cellular proteases are believed to contribute to ischemic myocardial injury. We assessed the potential of leupeptin (LEU), an inhibitor of lysosomal and cytoplasmic thiol-proteases, to interfere with proteolysis (PR) and reduce myocardial infarction (MI). Rats pretreated with LEU i.v. had 2 hr coronary ligation (L). Ventricular slices from normal (N) and ischemic (I) regions were incubated for 1 hr in anoxic Krebs-Ringer-HCO buffer (pH6.8) with cycloheximide 0.5mM. PR was measured as net tyrosine release (pmol/mg protein, mean ± SE):

	Control	LEU-1 (10mg/kg)	LEU-2 (40mg/kg)
N	426±39)n=23	306±25*(-28%,n=13)	247±23+(-42%n=5)
I	497±46(n=17)	284±38Δ(-50%,n=8)	134±37+(-73%n=6)

(*=p<.05,Δ=p<.01, +=p<.001 vs Control.

Three other groups of rats received saline, LEU-1 or LEU-2 10 min before 2, 4, 6, and 24 hrs after L. MI size, determined histologically 72 hrs after L, was not different among controls (mean 34±2%SE of left ventricle, n=30), LEU-1 group (33±4%,n=21) and LEU-2 group (37±2%,n=23). We conclude that 1) PR is not significantly increased in early MI and 2) even though LEU markedly reduces total PR in I regions, this action is insufficient to alter ultimate MI size. Thus, proteases inhibited by LEU (lysosomal cathepsins B, L, and H and cytoplasmic Ca-activated protease) do not appear to contribute to ischemic damage during acute MI.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01789-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Proteolysis in Normal and Ischemic Myocardium: Effects of Antipain, Pepstatin and Chymostatin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Roberto Bolli	Visiting Fellow	CB	NHLBI
Other:	Richard O. Cannon	Clinical Associate	CB	NHLBI
	Robert E. Goldstein	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Section on Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.5	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cellular proteolysis (measured as tyrosine production) was similar in slices of 2-hour ischemic and normal rat ventricular myocardium incubated in "ischemic" medium, suggesting that cellular proteases are not activated during acute myocardial ischemia. In vivo pretreatment with antipain (an inhibitor of lysosomal cathepsins A and B) reduced (-51%) proteolysis in ischemic (but not in normal) myocardium. Thus, antipain can be used to assess the role of cathepsins A and B in the development of ischemic cell death. In vivo pretreatment with pepstatin (an inhibitor of cathepsin D) reduced (-50%) proteolysis in normal (but not in ischemic myocardium, suggesting an importnt role of cathepsin D in myocardial protein turnover. Chymostatin (a weak inhibitor of cathepsins A, B and D) failed to inhibit proteolysis either in normal or ischemic myocardium.

Project description: Several studies suggest that cellular proteases (CP) contribute to cell death during acute myocardial ischemia (AMNI). To determine whether increased P occurs early after onset of AMI and to identify agents that reduce proteolysis (P) *in vivo* during AMI, rats were given i.v. saline or antipain (ANT) inhibitor of cathepsin A and B and of calcium-activated neutral protease, 12 mg/kg 20 min and 8 mg/kg 10 min before coronary artery ligation (L); 18 hrs and 1 hr before L, another group of rats received i.p. either dimethylsulfoxide (DMSO) 1 ml, pepstatin (PEP) (a specific inhibitor of cathepsin D) 8 mg/kg 1 ml DMSO, or chymostatin (CHY) (inhibitor of cathepsin A and B and of chymotrypsin-like protease) 60 mg/kg in 1 ml DMSO. Two hrs (ANT and saline groups) or 3 hrs (DMSO, PEP and CHY groups) after L, .5 mm thick slices were cut from ischemic (Isch) or normal (N) epicardium and incubated for 1 hr in Krebs-Ringer-bicarbonate buffer saturated with $90\%N_2-10\%CO_2$, pH 6.8, with cycloheximide (.5mM). The rate of P, assessed by net production of tyrosine by the slices (pmol/mg of tissue, mean ± SE), was:
(*=p<.02 vs DMSO; vs**=p.<001 vs saline; § = NS vs Nl)

	Saline	ANT	DMSO	PEP	CHY
Nl	66±5(n=23)	66±9(n=7)	40±6(n=12)	20±3*(-50%,n=5)	-
Isch	77±7(n=17)	§ 38±3**(-51%,n=5)	36±7(n=7)	25±8(n=6)	37±7(n=6)

This is the first demonstration that PEP reduces cardiac P in Nl, indicating an important role of cathepsin D in this process. Also, ANT markedly reduces P in Isch indicating that ANT-sensitive proteases account for a significant fraction of the total P occurring during AMI. Nonetheless, P is not increased after 2 hrs of AMI, suggesting that CP do not contribute to cell death during AMI.

Publications: Bolli, R., Cannon, R.O., Goldstein, R.E., Epstein, S.E.: Proteolysis in Normal and Ischemic Myocardium: Effects of Antipain, Pepstatin and Chymostatin. Clinical Research 28: 158A, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01790-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of Nifedipine and Diltiazem on Reperfusion Ventricular Fibrillation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Florence H. Sheehan	Clinical Associate	CB	NHLBI
Other:	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.25	.25

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The calcium-antagonist drugs nifedipine and diltiazem were studied for efficacy in preventing ventricular fibrillation during reperfusion following coronary artery occlusion. Neither of the drugs were effective. Nifedipine also does not inhibit calcium influx into myocardium during reperfusion.

Project Description: The efficacy of intravenous nifedipine, verapamil and diltiazem in preventing ventricular fibrillation during reperfusion after release of circumflex coronary artery occlusion was compared. Sixty dogs underwent open chest circumflex occlusion for 30 min, then abrupt reperfusion. Dogs were randomly assigned to control, low nifepidine (1 mcg/kg bolus over 3 min then 1 mcg/kg/min), high nifedipine (5 mcg/kg bolus over 3 min then 1 mcg/kg/min), verapamil (100 mcg/kg bolus then infusion dose yielding blood pressure fall of 10 to 20 mm Hg, mean 8.75 mcg/kg/min), or diltiazem (0.2mg/kg bolus then infusion adjusted to yield blood pressure fall of 10 to 20 mm Hg, mean 13.6 mcg/kg/min). Occlusion was not performed until blood pressure had stabilized at the dose given for at least 10 min.

During reperfusion, ventricular fibrillation occurred in 13/20 control dogs, 5/9 low nifedipine dogs, 6/8 high nifedipine dogs, 9/12 verapamil dogs and 10/11 diltiazem dogs.

To determine if nifedipine prevents the previously observed calcium influx into reperfused myocardium, 7 control and all nifedipine dogs were reperfused for 10 min. Hearts were quickly excised. Myocardium from normal and reperfused zones was lyophilized, digested and analyzed for calcium content using atomic spectrophotometry. The ratio of reperfusion zone calcium to normal zone calcium was 1.7 in control, 1.5 in low nifedipine and 1.9 in high nifedipine dogs.

Thus, nifedipine does not inhibit calcium influx during reperfusion. Nifedipine, verapamil and diltiazem do not protect against reperfusion ventricular fibrillation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01791-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Determinants of Arrhythmic Death Due to Coronary Spasm: Pre-Existing Coronary Stenosis Reduces Risk of VF Following Release of Coronary Occlusion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Florence Sheehan	Clinical Associate	CB	NHLBI
Other:	Stephen Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1/2	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The incidence of ventricular fibrillation during reperfusion of ischemic myocardium after release of coronary artery occlusion is reduced by 1) restriction of blood flow during reperfusion with a partial occluder or 2) increased collateral flow to the ischemic myocardium induced by chronic stenosis of an adjacent coronary artery. This suggests that the risk of ventricular fibrillation during relief of coronary spasm in patients may be greater in patients without coronary disease than in those with coronary disease.

PHS-6040
(Rev. 10-76)

Project Description: Coronary spasm may occur in the presence or absence of coronary disease (CAD). We therefore determined the effect of pre-existing coronary stenosis on the incidence of ventricular fibrillation (VF) during reperfusion (R) following circumnflex coronary artery (CFX) occlusion (O). In 20 open-chest dogs an occluder was placed on the CFX to partially restrict blood flow (F). The CFX underwent complete O for 30 min then abrupt R, leaving the partial occluder in place. During R peak F was 84±35% of baseline (mean +SD) in surviving dogs, and 156± 73% in dogs dying in VF ($p<.02$). In another 16 dogs, the LAD was gradually occluded by an ameroid constrictor; after 20 to 39 days, the CFX underwent acute O for 30 min then abrupt R. Collateral F to the CFX bed was measured by microspheres 10 min after O. Collateral F in dogs dying of R VF (N=9) was 27±28% of normal zone F, versus 58±22% in dogs (N=7) surviving ($p<.05$). Thus, risk of R VF is greater in dogs with normal coronary arteries than in dogs with either 1) a F limiting partial stenosis of the artery undergoing transient O, or 2) chronic stenosis of a second coronary artery providing collateral F. This suggests that the risk of VF during release of coronary spasm may be greater in pts without than in those with CAD.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01792-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Ventricular Septal Contour in Hypertrophic Cardiomyopathy: Two-Dimensional Echo Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Timothy P. Blair	Guest Worker	CB	NHLBI
	John S. Gottdiener	Head, Cardiology Consultation	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Clinical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .7	PROFESSIONAL: .6	OTHER: .1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two-dimensional echocardiography permits non-invasive assessment of ventricular septal contour. A variety of septal contours are present in patients with hypertrophic cardiomyopathy which are highly specific markers for that disease.

Project description: Hypertrophic cardiomyopathy is a disease of cardiac muscle in which the ventricular septum is usually involved prominently in the cardiomyopathic process. Because ventricular septum contour may have implications regarding diagnosis and advisability of operation, 70 patients with hypertrophic cardiomyopathy and 57 patients with other heart diseases or normal hearts were studied by two-dimensional echo using primarily parasternal long axis and apical views. A variety of contours were identified, with the most common (25 patients, 35%) being "lemon-shaped" (convex to both left ventricle and right ventricle). Ventricular septum contour in other patients were convex toward left ventricle and flat (30%) or concave (25%) to right ventricle, or flat toward left ventricle and convex to right ventricle (10%). In contrast, in each control patient the right and left septal surfaces were parallel. Patients with hypertrophic cardiomyopathy also showed variable distribution of ventricular septum hypertrophy. In most patients (59, 84%) hypertrophy involved all of ventricular septum and was maximal in the middle 1/3. Of note, in 8 (12%) of patients hypertrophy was discretely confined to the cephalad 1/3 of ventricular septum resembling a "tumor nodule", and in 3 (4%) patients was present only in apical regions. Hence: in patients with hypertrophic cardiomyopathy 1) a wide variety of septal contours are present that represent highly specific echo markers for that disease; 2) defining the distribution ventricular septum hypertrophic may be useful in the planning for septal myotomy and myectomy.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01793-01 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
24-Hour Ambulatory ECG Monitoring as a Predictor of Sudden Death in Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barry J. Maron	Senior Investigator	CB	NHLBI
Other:	Daniel D. Savage	Guest Worker	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cardiology Branch

SECTION
Clinical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.2	o

CHECK APPROPRIATE BOX(ES)
☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

"High-grade" ventricular arrhythmias are common in patients with hypertrophic cardiomyopathy. However, the presence (per se) of these arrhythmias was not predictive of sudden death in a population of patients followed for 3 years after the initial 24 hour ambulatory ECG monitoring.

PHS-6040
(Rev. 10-76)

Project description: Sudden death is a not rare sequelle of the natural history of patients with hypertrophic cardiomyopathy, although the mechanism of these catastrophic occurrences is unknown. Our previous 24 hour ambulatory ECG monitoring studies showed a particularly high prevalence (i.e., 66%), of high-grade "malignant" (e.g., Lown, grade 3 or higher) ventricular arrhythmias in 100 patients with hypertrophic cardiomyopathy (who were not taking cardioactive medications). Since the significance of these arrhythmias in identifying patients at high-risk for sudden death is not known, clinical outcome was followed prospectively in the 100 patients with previous 24 hour ECGs. After 3 years, 15 patients had undergone operation because of severe symptoms. Of the remaining 85 patients, 58 (68%) had had high-grade arrhythmias. Five pts died during follow-up and in 4, "malignant" arrhythmias were recorded. However, 54/58 (93%) of the pts with "malignant" arrhythmias survived the 3 yr follow-up period. We conclude that: 1) high-grade arrhythmias are commonly found in patients with hypertrophic cardiomyopathy by ambulatory ECG monitoring; 2) however, although such arrhythmias appear to identify most patients with hypertrophic cardiomyopathy at high-risk for sudden death; (e.g., high sensitivity), most pts with such arrhythmias do well (low specificity); 3) therefore, other (as yet undefined factors) must be present for ventricular instability in patients with hypertrophic cardiomyopathy to evolve into terminal arrhythmias.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01794-01 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Clinical Utility of Plasma Verapamil Levels in Patients with Hypertrophic Cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin B. Leon	Clinical Associate	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Taysir M. Jaouni	Research Associate	CH	NHLBI
	Henry M. Fales	Chief, Chemistry Branch	CH	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Chemistry Branch

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.1	OTHER: 0.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Plasma verapamil levels were measured using a highly sensitive essay employing high pressure liquid chromatography and fluorometric detection in 70 patients with hypertrophic cardiomyopathy on chronic oral verapamil therapy. There was marked interpatient variability in verapamil levels at each dosage and no differences between clinical responders, nonresponders, and patients with serious cardiac side effects related to verapamil. Plasma verapamil levels are of limited usefulness for monitoring therapy in patients with hypertrophic cardiomyopathy.

Project description: A highly sensitive, reproducible high pressure liquid chromotography assay was used to determine the importance of plasma verapamil levels (PVL) in guiding therapy of pts with hypertrophic cardiomyopathy (HCM). In 70 pts taking oral verapamil (V), there was marked variability between pts in PVL for each dosage.

dose (mg/day)	240	360	480	640
mean PVL±2SD (ng/ml)	84±72	163±172	174±208	258±170

In contrast, variability in peak and trough PVL for a given patient was relatively small (V peak/trough ratio for 23 pts=1.66±.07). PVL in 27 clinical responders (R), with improvement in NYHA functional class and >15% increase in exercise capacity were not different from 15 nonresponders (NR) (R=153±11, vs NR=175±18 ng/ml; NS). Moreover, PVL in 24 pts with serious electrophysiologic or hemodynamic side effects from V ranged from -30 to 540 ng/ml and the mean PVL (201±23 ng/ml) was no different from the R or NR. Thus, in pts with HCM on V therapy 1) there is marked interpatient variability in PVL, which may be due to differences in 1st pass hepatic metabolism 2) PVL are similar in R, NR, and pts with serious side effects. PVL are therefore of limited usefulness in predicting therapeutic or toxic effects in pts with HCM.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01795-01 CB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical Efficacy of Verapamil Alone and Combined with Propranolol in Treating Patients with Chronic Stable Angina Pectoris

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin B. Leon	Clinical Associate	CB	NHLBI
Other:	Douglas R. Rosing	Senior Investigator	CB	NHLBI
	Robert O. Bonow	Senior Investigator	CB	NHLBI
	Stephen E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cardiology Branch

SECTION

Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An in-hospital study involving 11 patients with chronic stable angina pectoris was undertaken to determine the effects of verapamil given alone and combined with propranolol. Patients improved exercise capacity with verapamil compared to placebo or propranolol and increased exercise time even further with the combination of verapamil and propranolol.

PHS-6040
(Rev. 10-76)

Project description: To determine the effectiveness of oral verapamil (V) alone and with propranolol (P) in pts with chronic angina pectoris (AP), upright bicycle exercise testing (ExT) was performed in 11 pts (9 refractory to β-blockers), 29-62 yrs, in an inpatient, single-blinded crossover study. Compared to placebo (PL), V (480 mg/d) improved exercise time (ET) in all pts (from 307±40 to 508±42 sec; $p<.001$) and was more effective than P (160-320 mg/d) (385±34 sec; $p<.005$ vs V). V+P further increased ET (to 590±63 sec; $p<.01$ vs V) and 9/11 pts (82%) were pain-free during ExT (in contrast to 2/11 with P and 1/11 with V). Time to 1 mm ST-segment depression was increased by both V (from 178±21 to 358±50 sec; $p<.05$) and V+P (476±93 sec; $p<.05$) but not P. At the workload causing angina when on PL, V decreased heart rate (HR) (from 112±3 to 104±3; $p<.05$) and tended to decrease pressure-rate product (PRP) (from 18,260±780 to 15,930±940; $p<.1$) while V+P further decreased HR (to 76±3; $p<.001$) and PRP (to 9,660±510; $p<.001$). Training effect was absent, as determined by comparable ExT on no medications, before study, during PL and after study. Thus, in this study of pts with stable AP (1) V was a more effective anti-anginal agent than P and (2) V+P was well tolerated and provided additional improvement in exercise capacity over either drug alone.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01796-01 CB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Effects of Varying Doses of Verapamil on Hemodynamics and Myocardial Collateral Blood Flow

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	N. J. Davenport	Staff Fellow	CB	NHLBI
Other:	R. E. Goldstein	Senior Investigator	CB	NHLBI
	R. Bolli	Visiting Fellow	CB	NHLBI
	M. Leon	Clinical Associate	CB	NHLBI
	R. Aamodt	Nuclear Medicine, Clinical Center	NM	CC
	S. E. Epstein	Chief, Cardiology Branch	CB	NHLBI

COOPERATING UNITS (if any)
Nuclear Medicine, Clinical Center

LAB/BRANCH
Cardiology Branch

SECTION
Experimental Physiology and Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.5	.4	.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Verapamil, an investigational drug useful in cardiovascular disease, has been shown in animals to be beneficial following myocardial infarction. We investigated the effects of stepwise incremental doses of verapamil on heart rate, blood pressure and myocardial collateral blood flow in dogs. Blood flow to normal heart tissue increased with low doses of verapamil that did not change heart rate or blood pressure. Higher doses of verapamil increased normal tissue flow further, but also produced an increase in heart rate without blood pressure changes. Blood flow to the ischemic myocardium was not altered by any dose of verapamil tested.

Project description: Verapamil (V) has been thought to reduce myocardial damage following coronary occlusion (O). To clarify effects of V on coronary and systemic hemodynamics we gave increasing amounts of V to 8 closed-chest dogs beginning 5 min after O. V doses were administered as a bolus (.025. .05, .1 and .2 mg/kg) followed by an infusion that maintained steady blood levels (mean peak 144 ng/ml). Pre-V and 20 min after each bolus we measured myocardial blood flow (BF; microspheres), heart rate (HR), blood pressure (BP), cardiac output (CO), and total peripheral resistance (TPR). Nine control dogs (C) received saline. Mean data were as follows (NZ=normal zone BF, IZ=ischemic zone BF, epi=epicardium, endo=endocardium):

	NZ Epi/Endo ml/min/g	IZ Epi/Endo ml/min/g	HR bpm	BP tor	CO L/min	TFR dsc
1)V.05	1.2* /1.2	.36/.25	81	92	3.0	2657
C	.97/ 1.1	.33/20	82	84	3.0	2277
2)V.2	1.3* /1.3*	.35/.23	93*	86	3.1	2334
C	.81/ .90	.41/.21	77	79	3.0	2206

*$p<.05$ vs matched C; $p<.05$ vs pretreatment base V.2 raised HR but BP, CO and TPR were unchanged. V increased NZ BF, epi >endo, even in doses insufficient to raise HR or TPR. However, no V dose produced a consistent rise in collateral BF. Thus, in closed-chest dogs with O, V increased perfusion of normally supplied myocardiaum but did not alter perfusion of ischemic regions.

Publications: None

Annual Report of the
Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

The Membrane Enzymology Section, under the leadership of Dr. Richard Hendler, is studying the electron transport chain in bacterial and mammalian membranes in order to identify all of the components involved in the passage of electrons from the initial substrate to O_2 and to elucidate the mechanism by which the electrical energy is converted into a form that the cell can utilize. The Organelle Biochemistry Section, headed by Dr. Martin Flavin, is primarily involved with the unique reaction in which a tyrosine residue is added to the carboxyl terminus of the α-chain of tubulin and the role of this reaction in the function of microtubules in the mammalian cell. The Cellular Physiology Section, which Dr. Evan Eisenberg leads, is elucidating each of the intermediate steps in the hydrolysis of ATP by muscle actomyosin, the regulation of this reaction by tropomyosin and troponin, and, with the collaboration of Dr. T. Hill (NIAMDD), modelling these reactions in a way that enables the quantitative correlation of the biochemical events to the physiological events in the living muscle. The Cellular Biochemistry and Ultrastructure Section, under the leadership of Dr. Edward D. Korn, is studying the biochemical basis of cell motility including the mechanism of polymerization of actin and its regulation by cellular proteins and the properties and regulation of myosin isoenzymes of non-muscle cells.

(1) Membrane Enzymology Section: The automated electrodic oxidizing system developed previously to study the path of electrons in *E. coli* membranes was modified to include a pH stat. This led to the discovery that the transfer of 4-8 electrons between about 320 and 330 mV is accompanied by the release of protons. From the redox potential of the membrane carrier and its electron proton magnetic resonance signal in its oxidized state, the active protein has been identified as one of a unique class of high potential iron-sulfur proteins. This is the first evidence for such a protein in an electron transport chain and for its involvement in respiration-linked acid production.

A fully active form of this high potential iron-sulfur protein can be solubilized from the membrane by 0.4% deoxycholate and separated from cytochrome oxidase with which it may be coupled in the intact membrane. Upon ultrafiltration, both the filtrate and the concentrated protein are inactivated but full activity is recovered when the two fractions are mixed. The ultrafiltrate can be replaced either by material extracted from it by chloroform-methanol, i.e. lipids, or by a mixture of Tris and deoxycholate. Variable activity of the reconstituted soluble material can be stabilized by the addition of Cu^{2+} which is not required when membranes are used.

It is clear from the time courses of the changes in voltage in the membrane preparation and the release of protons that the acidificaiton is tightly coupled to electron transport, i.e. to the oxidation of the endogenous electron donor. However, the magnitude of proton release is too great for any membrane component to be the source. The only possibility seems to be the phosphate ions in the buffer solution.

In related studies, Dr. Hendler and his collaborators have been able to solubilize the multienzyme complex succinoxidase, which carries electrons from succinate to O_2 through multiple intermediates, and to reconstitute it functionally into artificial membranes. The reconstituted system has recovered its energy transducing properties in that a proton gradient is developed across the membrane. However, only about 10% of the number of protons is ejected per atom of oxygen reduced compared to the activity of the native membranes. In order to obtain reconstituted membranes with higher efficiency, new methods of preparation will be employed and attempts will be made to separate the preparations into more active and less active sub-populations.

To measure the very small amount of acidificaiton produced by the reconstitutes succinoxidase system, it was necessary to develop, in collaboration with Walter Friauf of BEIB, a new type of ΔpH meter capable of measuring changes of a few ten-thousandths of a pH unit per minute.

(2) Organelle Biochemistry Section: Previous reports from this group have shown that the tyrosylating enzyme that adds tyrosine to the carboxyl terminus of α-tubulin does not work on polymerized microtubules but prefers the tubulin dimer. In contrast, new results show that the highly purified detyrosylating enzyme has a marked preference for the polymerized microtubule. This behavior of the two enzymes suggested that tyrosylation and detyrosylation might be related to the interconversion of tubulin dimer and polymer. One way to study this is to examine the steady state equilibrium between tubulin dimer and microtubule polymer using tyrosylated and detyrosylated material. According to current belief, there is an exchange of tubulin dimer at both ends of a microtubule with dimer in solution. However, at one end there are more on-reactions than off-reactions while at the other end there is a corresponding excess of off-reactions over on-reactions. The consequence is a net addition of tubulin dimer at one end of the microtubule and an equivalent net loss of tubulin dimer at the other end. With no change in the concentration of tubulin dimer or microtubule, there is a flow (treadmilling) of tubulin dimers through the microtubule. Careful study has thus far revealed no difference in the treadmilling rates of tyrosylated and detyrosylated tubulin.

In the cell, and in purified microtubule preparations, there are several high molecular weight microtubular associated proteins (MAPS). MAP_1 has a molecular weight of about 360,000 and MAP_2 of about 300,000. MAPS are necessary for the polymerization of tubulin dimer; MAP_2 is most effective. Both tyrosylated and detyrosylated tubulin can be freed of MAPS by chromatography on phosphocellulose. No differences in their rates of assembly were detected when mixed with either the mixture of MAPS or MAP_2. However, about 25% more MAPS seemed to bind to microtubules polymerized from detyrosylated tubulin than when tyrosylated tubulin was used.

The interaction of tubulin with phospholipid vesicles has also been studied. This interaction has been found not to be affected by the state of tyrosylation of the tubulin dimer but the reaction is nevertheless of interest as a possible model system for the postulated interaction of tubulin or microtubules with biological membranes. When added to the lipid bilayers at their transition temperature, tubulin dimer binds strongly to the vesicles as monitored by the induction of leakage of vesicle content. The bilayer itself remains intact and the tubulin seems, at least in part, to be associated with the hydrophobic interior of the bilayer to which it has access only during the phase transition.

A possible role for tyrosylation has come from studies of chemotaxis of leukocytes induced by the tripeptide formylmethionylleucylphenylalanine.

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Although control and stimulated cells seemed to have the same content of tyrosine ligase and the same proportion of tyrosylated tubulin, there was a 2-3 fold stimulation of the turnover of the C-terminal tyrosine upon stimulation. The effect was not due to increased transport of tyrosine and was tightly coupled to the chemotactic stimulation being blocked by four inhibitors that act at four different sites. Of course, it is not yet known if this tyrosylation is functionally related to the chemotaxis but the coincidence of effects is intriguing.

(3) Cellular Physiology Section: The traditional view of the actomyosin ATPase cycle holds that ATP dissociates the actomysoin complex when it binds to myosin and that the release of the hydrolytic product, Pi, occurs from dissociated myosin. At that point the actin and myosin rebind to each other. The physiological equivalent is thought to be the cycling of cross-bridges between the thin filaments (actin) and the thick filaments (myosin) in the muscle. Several years ago, Dr. Eisenberg and Dr. Kielley proposed that the rate-limiting step in this reaction was the conversion of myosin from a refractory state, that was incapable of binding to actin, to a non-refractory state, which could bind to actin. This could account for the experimental observation that at 5^{o} most of the myosin was not bound to actin even at very high actin concentrations approaching Vmax for the actomyosin ATPase. Last year, Dr. Eisenberg and his associates, in order to explain newer data obtained at more physiological temperatures, proposed that, although the refractory and non-refractory states do exist, myosin was always bound to actin during the ATPase cycle. Rather than cycling between bound and unbound states, according to this new model, myosin cycled between states weakly bound to actin and states strongly bound to actin. The weakly bound states would be those in which ATP or its products, ADP and Pi, were bound to the myosin; the strongly bound states would be when ADP or no nucleotide were bound to the myosin. The refractory to non-refractory transformation would occur while the myosin was bound to the actin. This newer model can be schematized as follows:

$$\begin{tikzcd}
M \arrow[r, shift left] \arrow[d, shift right] & M\cdot ATP \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & M\cdot ADP\cdot Pi_R \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & M\cdot ADP\cdot Pi_N \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & M\cdot ADP \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & M \arrow[l, shift left] \arrow[d, shift right] \\
A\cdot M \arrow[r, shift left] \arrow[u, shift right] & A\cdot M\cdot ATP \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & A\cdot M\cdot ADP\cdot Pi_R \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & A\cdot M\cdot ADP\cdot Pi_N \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & A\cdot M\cdot ADP \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & A\cdot M \arrow[l, shift left] \arrow[u, shift right]
\end{tikzcd}$$

where M = myosin and A = actin. The validity of this model has been directly tested by assaying the effect of increasing actin concentration on the rate and magnitude of the Pi burst (the initial release of bound Pi from myosin upon the addition of actin before the steady state rate of hydrolysis occurs). According to the older theory, at very high actin concentrations the rate of the initial burst would be slightly decreased or not affected while the magnitude of the burst would be greatly diminished. The experimental observations were that the rate of the burst increased markedly while its magnitude was only slightly diminished. Mathematical modelling shows that these results can be accounted for by the new kinetic model. It should now be possible to proceed to measure the binding constants between actin and myosin in its several states to obtain the final proof for the new model.

In the physiological situation, the actomyosin ATPase is regulated by the tropomyosin-troponin complex which binds to F-actin filaments and renders the ATPase Ca-dependent. This occurs by either a steric blocking of the actin or a conformational change in the actin such that myosin cannot bind to the actin until Ca is added to reverse the block. Studies now show that the binding of myosin-ADP to the actin-tropomyosin-troponin complex is much weaker than its binding to actin alone at low concentrations of myosin but significantly tighter at high myosin concentrations. Thus, the ratio of the binding constants changes from about 1:100 to about 3:1. This shows a marked positive cooperativity of myosin binding in the presence of tropomyosin-troponin and no cooperativity in the absence of tropomyosin-troponin.

In contrast to these results with myosin-ATP, the binding of myosin-ATP to actin was largely unaffected by the presence of tropomyosin-troponin (only about 2-fold weaker in the presence of Ca than in its absence) while the ATPAse rate was greatly affected (only about 6% in the absence of Ca of the rate in the presence of Ca). These data, combined with those discussed in the previous paragraph, suggest that the primary effect of Ca-tropomyosin-troponin is to weaken greatly the binding of myosin-ADP while not affecting the binding of myosin-ATP. This would have the effect of inhibiting the release of Pi, i.e. of inhibiting the conversion of myosin-ATP to myosin-ADP in the actomyosin complex.

(4) Cellular Biochemistry and Ultrastructure Section: A number of observations have led Dr. Korn and his associates to propose a modification of a theory for actin polymerization recently proposed by a German scientist, Albrecht Wegner. (1) At concentrations very much lower than the actin concentration, cytochalasin D inhibits the rate of polymerization of actin and, when added to polymerized actin at steady state, causes partial depolymerization of the actin. (2) A complex of spectrin/actin isolated from red blood cells accelerates the initial rate of actin polymerization. (3) The rate of polymerization of actin nucleated by spectrin/actin complex is inhibited by cytochalasins but cytochalasins do not cause depolymerization of actin when the polymerization has occurred in the presence of spectrin/actin complex. (4) Cytochalasins uncouple the hydrolysis of ATP by actin from the polymerization of actin and greatly stimulate the hydrolysis of ATP by actin in the monomeric state. (5) As first demonstrated by other laboratories, cytochalasins bind to actin filaments (approximately one molecule of cytochalasin per filament). (6) Under certain circumstances, cytochalasins accelerate the initial rate of actin polymerization while inhibiting the final extent of polymerization.

According to Wegner's model for actin polymerization, the slow nucleation step (in which several actin monomers form a very short actin oligomer) is followed by an elongation step in which, in the presence of ATP, actin monomers add to both ends of the actin filament but at different rates. Dr. T.D. Pollard has obtained direct visual evidence for this in electron microscopic studies. Two reactions will occur at both ends of the growing filament: the reversible conversion of monomeric actin-ATP to polymeric actin-ADP and the reversible dissociation of polymeric actin-ADP to monomeric actin-ADP. In the presence of excess ATP, actin-ADP will be converted to actin-ADP because actin has a higher affinity for ATP than for ADP. Polymerization will cease and a steady state will be reached when the net rate of addition of actin monomers to both ends of the filament equals the net loss of actin monomers from both ends of the filament. Wegner proposed that the essentially irreversible hydrolysis of ATP allows, but does not require, an asymmetry in the process so that there can be net addition of monomers at one end of the filament with a numerically equivalent net loss of monomers from the other end. Under these conditions, the concentrations of actin polymer and monomer will remain constant but actin subunits will, on average, treadmill through the filament.

Our data can be explained by, and therefore provide support for, this model as follows. Cytochalasins bind to the normally more rapidly growing end of the actin filament and inhibit the rate of polymerization by restricting filament elongation to the normally more slowly growing end. When added to actin filaments at steady state, cytochalasins cause depolymerization by effectively blocking events at the net addition end while depolymerization continues at the net depolymerization end until it reaches a true equilibrium. Spectrin/actin complex accelerates actin polymerization by providing nucleating centers thus by-passing the rate limiting nucleating step. Cytochalasins inhibit polymerization induced by spectrin/actin complex by blocking events at the growing end but do not cause depolymerization of the filament because the normally net depolymerizing end is now blocked by the spectrin/actin complex. Under some circumstances, cytochalasins can stimulate the initial rate of polymerization by accelerating the formation of nuclei by binding to, and stabilizing, one end of the actin oligomer. When monomer concentration is still high, the rate of elongation from the less favored end of the nuclei is still greater than the rate of nucleation. The effects of cytochalasins on the hydrolysis of ATP by actin and the uncoupling of this hydrolysis from actin polymerization can be explained by a modification of the model. It is assumed that monomeric actin-ATP is in equilibrium with monomeric actin-ADP-Pi and that this latter species is the monomeric form that adds to the growing filament end. The Pi that is released during polymerization would, thus, be derived from the bound Pi at the end of the filament. There would also be a slow breakdown of actin-ADP-Pi to actin-ADP + Pi. Cytochalasins would greatly accelerate this ATPase activity of monomeric actin by binding to the actin and displacing the ADP and Pi, being in turn replaced by ATP. The inability to demonstrate binding of cytochalasins to monomeric actin, in the presence of excess nucleotide, would be expected if monomeric actin had a much greater affinity for ATP than for cytochalasins. Details of these models are presented in Project Report No. 00401-06.

The other major area under investigation by Dr. Korn and his colleagues is the characterization of the three myosin isoenzymes of Acanthamoeba castellanii. It had previously been demonstrated that this amoeba contains two single headed myosins (IA and IB) which have heavy chains of relatively low molecular weights (130,000 and 125,000 respectively) and a two headed myosin (II). By the use of specific antibodies raised against the isolated heavy chains of the three enzymes (isolated by SDS polyacrylamide gel electrophoresis) it has now been demonstrated that (1) by this additional criterion the three myosins are true isoenzymes; (2) no related polypeptides of higher molecular weight can be detected in the cell so that the isolated enzymes are probably the native enzymes; (3) all cells in a culture contain all three enzymes; (4) the myosin I isoenzymes and the myosin II isoenzyme are differentially localized within the cell with myosin IA and IB being relatively more concentrated neither the cell periphery. With these antibodies it has also been possible to show that a related strain of Acanthamoeba castellanii and two different amoebae, Acanthamoeba astronyxis and Nigleria gruberii, contain similar myosin isoenzymes. These remain then the only known examples of non-muscle cells that contain myosin isoenzymes and the only examples of myosin molecules of the unusual low molecular weights characteristic of myosins IA and IB.

Previous work had s that actin can activate the ATPase activity of myosins IA and IB only when their heavy chains are phosphorylated. This is different from the situation for smooth muscle and vertebrate nonmuscle cells in which phosphorylation of one of their light chains is required for actomyosin

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ATPase activity. Therefore, in myosins IA and IB both the ATP-site and the regulatory phosphorylation site are on the heavy chain. It has been shown now that the region of the heavy chain that is phosphorylatable by the specific myosin I heavy chain kinase can be removed by subtilisin digestion and that full enzymatic activity is retained including actomyosin ATPase. Therefore, the nonphosphorylated site is a repressor of actomyosin ATPase activity and this inhibition can be derepressed by either phosphorylating the site or removing the site by controlled proteolytic degradation.

It has also been possible to label covalently the ATP-site by exposing myosins IA and IB (as well as myosin II and muscle myosins) to fluorescent light in the presence of ATP. This is the first time this technique has been applied to myosins. Proteolytic degradation of the 130,000-dalton chain of myosin IA after covalently labeling the ATP-site and the phosphorylation site led to the conclusion that both sites are retained in a 115,000-dalton peptide which is then cleaved to an 87,000-dalton peptide that contains the phosphorylation site and a 28,000-dalton peptide that contains the ATP-site.

Previous preparations of myosin II were not actin-activatable. A preparation has now been obtained whose ATPase can be activated by actin. Careful analysis of the two forms of myosin II revealed the only difference to be that the nonactin-activatable forms contained nearly 4 mol of P per mol of myosin while the actin-activatable forms contained about 2 mol of P per mol of myosin. Autoradiography of acrylamide gels of myosin isolated from cells grown in the presence of [^{32}P]Pi showed that all of the phosphate was contained in the heavy chains of myosin II. It was then discovered that actin-activation of all forms of myosin II was greatly enhanced by dephosphorylation of the myosin by either commercially obtained potato acid phosphatase or a smooth muscle myosin phosphatase. Myosin II is, therefore, the first example of a myosin whose ATPase is actin-activated only when the molecule is dephosphorylated and the first example where 2 phosphorylation sites per heavy chain may be involved. At this time, the Acanthamoeba is by far the best enzymatically characterized nonmuscle motile system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00401-14 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Potentiometric studies of respiratory components of E. coli and rat liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard W. Hendler	Head, Sec. on Membrane Enzymology	LCB NHLBI
Other:	Richard I. Shrager	Mathematician	LSMM DCRT
	Hideo Kon	Physical Chemist	LCP NIAMD

COOPERATING UNITS (if any)
Laboratory of Statistical and Mathematical Methodology, DCRT
Laboratory of Chemical Physics, NIAMD

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new kind of potential respiratory component has been found in E. coli membranes. It is analogous to cytochrome oxidase in that its midpoint potential and multiplicity for electron transfer are very similar. It can be physically separated from cytochrome oxidase by extraction with deoxycholate. Its optical and electronparamagnetic properties identify it as a high potential iron sulfur protein or HIPIP. It appears to require copper and lipid for its proper function which involves the oxidative generation of free acid. All indications point to phosphate buffer as the source of the protons.

PHS-6040
(Rev. 10-76)

Background: The previous report described the oxidative release of acid in a suspension of *E. coli* membranes. The release appeared to occur in two phases with a 4 electron transfer in the region of ∿315 mV to 340 MV and a 2 electron transfer from ∿340 mV to ∿400 mV. It was also found that a spectral change representative of the class of iron-sulfur proteins accompanied this process. The present report provides much more information on these phenomena.

Major findings: Because iron-sulfur proteins are notoriously labile to acid, the automated electrodic oxidizing system used in this work was modified to include a pH stat which introduced measured amounts of sodium hydroxide as needed to keep the pH near neutrality. Under controlled pH conditions, it was found that the acid release phenomenon was really monophasic; the slowing down phase previously seen at higher voltage reflected the moving of the system away from its optimal pH. The actual range during which all of the acid is released is between ∿320 and ∿330 mV, indicating a 4 to 8 electron transfer process. There is a unique class of iron sulfur proteins, called high potential iron sulfur proteins or HIPIPS which has redox potentials at about 330 mV. The HIPIPS differ from other iron sulfur proteins in that they are paramagnetic in the oxidized state and their EPR signal is found at $g \approx 2.06$ rather than at $g \approx 1.94$. We established that our active protein was a new kind of HIPIP. A pronounced EPR signal at $g = 2.06$ arose on oxidation in the same narrow voltage range that elicited acid release and the spectral change. The aspects which are entirely unique to our HIPIP are: its ability to transfer 4 to 8 electrons, its apparent involvement in respiration-linked acid generation, its location in an electron transport chain, its unusual EPR saturation behavior, and its possible interaction with copper (to be described below). We have been successful in finding a way of releasing the system from the membranes in a fully active form by extraction with deoxycholate (DOC) at 0.4% concentration. This separates the HIPIP from the cytochrome oxidase with which we suspect there is a direct interaction in the membrane. When the DOC extract is concentrated by filtration through an Amicon PM 10 filter, it is found that the concentrate and filtrate are each inactive, but fully active when re-mixed. The two fractions can be stored frozen indefinitely at -86°C. The activity of the filtrate was lost upon fractionation on Dowex 1. The chloroform-extracted lipids, however, were active. Surprisingly, Tris buffer can partially stimulate the PM 10 concentrate and this effect is greatly enhanced if DOC is added with the Tris. Another very good substitute for the PM 10 filtrate of the DOC extract, is a cell supernate that has not been treated with DOC. It thus appears that the system has a lipid requirement that can be partially satisfied by DOC in the presence of Tris. A unique aspect of the controlled pH oxidations using the extracted system is that voltage control in the critical 320 to 330 mV region is very much more difficult than that with the membrane-bound system. In fact it is sometimes impossible. It was learned that the addition of a low amount of Cu^{++} (i.e., ∿40 μM) brought the system under control. Cu^{++} was acting as a direct participant in the reactions rather than as a simple redox buffer because higher concentrations which provided excellent buffering killed the acidification response. These observations are extremely interesting because copper is an essential component of mammalian cytochrome oxidase but its function is totally unknown. The redox properties of the HIPIP studied here are very close to those of the cytochrome oxidase in terms of redox potential and number of electrons transferred. When the HIPIP is in the membrane, no copper has to be added for voltage control and concommitant acidification. Although E. coli cytochrome oxidase has not been purified and the presence of Cu^{++} established, our EPR studies show that a copper-like signal does arise as we oxidize this component. A time-course study of the rate of acidification and of voltage changes indicates a strong correlation between the supply of reducing equivalents by the bacterial extract and the release of acid. This provides evidence that the acid release is strongly coupled to the oxidation of the endogenous electron donor. Calculations of the amount of acid generated in terms of the amount of bacterial material present show that the protons are not supplied from a donor source provided by the bacteria. The only component present which has sufficient protons available is the phosphate buffer.

Increasing the concentration of phosphate increases acid generation and replacing most of the phosphate with other buffers decreases acid production. We are trying to obtain more direct evidence that the system provides free acid by a process which separates protons from phosphate.

Proposed course of project: We plan to continue our studies of the acid-producing phenomonon in order to isolate the component involved and to investigate its possible role in the conversion of redox energy to metabolically usable forms. We also plan to study purified mammalian mitochondrial cytochrome oxidase to see if evidence for multielectron transfers can be demonstrated with this enzyme.

Publications:

Hendler, Richard W., and Shrager, Richard I.: Potentiometric Analysis of Escherichia coli Cytochromes in the Optical Absorbance Range of 500 nm to 700 nm. J. Biol. Chem. 254: 11288-11299, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00402-08 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

DNA synthesis in E. coli.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard W. Hendler	Head, Sec. on Membrane Enzymology	LCB NHLBI
Other:	Raymond Scharff	Physiologist	LCB NHLBI
	Musetta Hanson	Staff Fellow	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Membrane Enzymology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Factor "E" involved in the formation of a DNA synthesizing complex appears to be associated with membranes. It can be released and activated by detergent. Commercial DNA polymerase I, although it contains only a single polypeptide, exists in two separable forms. Only one of these is involved in complex formation. The 5'→3' exonuclease portion of DNA polymerase I, which is essential for cell viability, is also essential for complex formation from DNA polymerase, when "E" is added.

PHS-6040
(Rev. 10-76)

Background: The previous report established that the bulk of natural complex involving DNA polymerase I and the rec BC enzyme is found concentrated in the membrane fraction of *E. coli*. A new factor, "E", was also described which was essential for holding complex together during isolation and which, when added to commercial polymerase I, could form complex. The present report confirms and extends these observations.

Major findings:

A. "E" exists in the cell in a membrane-bound form.
Evidence: (1) It is sedimented in a centrifugal field with the membranes. (2) It is found in the void volume of a gel column which excludes molecules > 50 x 10^6 daltons. It is also excluded from a 3% agarose-acrylamide electrophoresis gel. (3) It is released into a 100Kg supernatant fraction and also markedly stimulated when the particulate material is treated with deoxycholate. The activity is also stimulated by Triton x-100 in the electrophoresis gel or in the assay cocktail. (4) Detergent released "E" mixed with polymerase I shows far less activity in the pocket of an 8% acrylamide electrophoresis gel and much more activity running included.

B. Commercially purified *E. coli* DNA polymerase I contains two forms of enzyme. Only one of these forms is competent to form complex when treated with "E".
Evidence: (1) Polymerase I in PAGE (i.e. polyacrylamide gel electrophoresis) shows two active components at Rf ∿0.62 and ∿.71. (2) Natural complex shows one active component at Rf ∿0.54. (3) Upon further purification, natural complex dissociates yielding an active polymerase product which runs exactly to the position of the slower of the two polymerase spots. This material could then be converted back to complex by treating it with "E". (4) Adding "E" to polymerase I also forms the component which migrates at ∿0.54. This appears to be accompanied by the loss of the slower of the two polymerase spots. (5) When polymerase I is subjected to PAGE at a concentration where no activity is seen with native DNA, adding "E" to the gel assay cocktail reveals activity at the position of the slower spot of polymerase I. When Triton and DOC are present in the PAGE system, polymerase I again shows two active components. However, the slower component now is found at Rf ∿0.27 and the faster at ∿0.62. Natural complex in this system is slowed to about Rf 0.33. (6) Adding increasing amounts of "E" to a fixed amount of polymerase I leads to a disappearance of the slower polymerase spot and a concommitant appearance of complex at Rf ∿0.33. (7) The competent form of polymerase represents only a small modification of the molecule. This is because in SDS PAGE only one predominant peptide is seen. The alteration is most likely one of conformation, state of aggregation or presence of an adjunct which can markedly retard its migration in the detergent-containing PAGE. A likely candidate for this latter possibility is a lipid. (8) Further light on the requirements in the polymerase molecule which allow it to be converted to complex is the observation that a large fragment formed from the molecule by limited proteolysis can not be converted to

complex. This large fragment contains 70% of its molecule, all of its polymerase activity and all of its 3'→5' exonuclease activity. It is most interesting that the loss of the small fragment which contains the 5'→3' exonuclease activity, which is the only part of polymerase I that is absolutely essential for viability, also prevents the formation of complex.

C. A gel electrophoresis eluting, concentrating and recovery apparatus was developed which utilizes the existing disc PAGE apparatus. It is possible now to run a thick preparative gel, cut out the band of interest and recover the active material in a collection cup. This cup can then be fit in a flow dialysis apparatus to change its buffer, if desired, for subsequent work-up.

Proposed course of project: Because of growing requirements for additional help on the other major activity in the section, namely a study of bioenergetics, the effort expended on the DNA problem will be decreased. Nevertheless, we will try to explore the nature and functions of this intriguing biological complex involving DNA polymerase I, and also the mechanism of action of "E".

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 00404-21 LCB

PERIOD COVERED

October 1, 1979-September 30, 1980

TITLE OF PROJECT (80 characters or less)

Further characterization of the large fragment from the α-chain of fibrinogen (A or P1 peptide)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:

Elemer Mihalyi	Research Chemist	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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1	1	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The main purpose of this work was to prove that the C-terminal segment of the α-chain of fibrinogen, which is removed by plasmin, is not a random coil, but possesses a tight structure.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Generally, it was assumed that the C-terminal segment of the α-chain of fibrinogen, which is removed very fast by plasmin (A-peptide or P1 peptide), is a polypeptide in a random coil conformation. Doubt was cast on this by the presence of Lys and Arg residues in this segment which are not susceptible to trypsin or plasmin attack. In the present studies hydrodynamic measurements were performed to establish the shape of this fragment, as well as its unfolding in the presence of denaturing agents.

Methods Employed: Sedimentation velocity, sedimentation equilibrium, viscosity, gel filtration chromatography.

Major Findings: All the hydrodynamic data suggest that the large C-terminal α-chain fragment of fibrinogen is present as an elongated particle with an axial ratio of about 1:10. The intrinsic viscosity in nondenaturing solvents is 9 ml/g and is increased to about 16 ml/g in the presence of 5 M guanidinium HCl. This clearly shows a transition from a tight to a looser conformation. However, the value in guanidine is lower than expected for a random coil of 24,000 molecular weight. Possibly this is caused by the peculiar sequence of this chain, for which guanidine is not as good a solvent as for the usual polypeptide chains.

Trypsin cleaves the fragment approximately in two equal parts with a concomitant reduction of the asymmetry of the original particle. This suggests that the latter had two domains connected by a loose segment accessible to proteolytic enzymes.

Significance to Biomedical Research: The P1 peptide has an unusual sequence and a peculiar conformation. These were preserved in the molecule probably because they have a function. The significance of this work will depend naturally on finding this function.

Proposed Course: Further work will be directed mainly to finding the biological function of the segment of the fibrinogen molecule which is removed as peptide P1 in the early phase of the proteolytic fragmentation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00405-06 LCB

PERIOD COVERED
October 1, 1979-September 30, 1980

TITLE OF PROJECT (80 characters or less)
Circular dichroic studies of protein denaturation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Frederick H. White, Jr.	Senior Investigator	LCB	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is well known that development of conformational structure depends on amino acid sequence, although the exact relationship, which would make possible a reliable prediction of native conformation from a knowledge of primary structure, remains unclear. The native conformation develops from the randomly coiled chain of the denatured (or newly synthesized) protein through the influence of many interactive forces, including "nucleation", whereby a transition to the native conformation occurs concommitantly with formation of SS bonds. The role of SS bonds in this process is not well understood, but it is generally assumed that they have a stabilizing function, rather than a positive influence on structure formation. Recent work in this laboratory questions this assumption, and further work is in progress to clarify the role of SS bonds in chain folding.

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Project description

Objective: To examine conformational structures remaining in denatured proteins as they relate to the folding process.

Methods employed:

(1) The Cary-60 Recording Spectropolarimeter was used for all studies of circular dichroism (CD). Processing of CD data was done with the PDP-10 Computer, employing the "MLAB" system of curve fitting.

(2) Methods developed in this laboratory have been used for partial and complete reduction of SS bonds.

(3) Amino acid analyses (to determine the reduction level by measuring the S-carboxymethylcysteine content of the carboxymethylated reduced protein) were carried out on a Beckman Model 120 automatic amino acid analyzer.

(4) Protein concentrations (for the purpose of determining extinction coefficients of partially reduced samples) were determined by the method of Lowry (J. Biol. Chem. 193:269 (1951)).

(5) Gel filtration columns (Bio-Gel P-60) have been employed as a means of differentiating between monomeric and polymeric forms of partially and fully reduced proteins under a variety of experimental conditions.

Major findings: Earlier (Project No. Z01 HL 00405-05 LCB), experimental observations were described, which were compatible with the concept that native SS bond formation was a prerequisite for the normal course of conformational development within the fully reduced (or newly synthesized) protein.

In a further study of lysozyme reduction under non-denaturing conditions, the ratio of chain length fraction as alpha-helix to that present as beta structure, showed a marked decrease at the half-reduced level with no significant difference from that point to full reduction. This is a further indication that transition from native to denatured structure occurs with cleavage of the second SS bond.

A detailed study has been made on the effects of partial reduction on the extinction coefficient of lysozyme. This value is necessary for an accurate calculation of protein concentration, which, in turn, must be known for determination of ellipticity values. Also, additional information can be obtained on the nature of the structural changes that occur with reduction.

Thus, the native value for $E^{1\%}_{1\,cm}$ at 280 mn (27.1) remains nearly unchanged until the three-fourths reduction level is reached, and then falls to the fully denatured extinction of 22.7 as full reduction is approached. While no significant difference is seen between these levels by CD, which measures secondary structure, there remains a tertiary interaction, probably involving hydrogen bonding of tyrosine, to account for the persistence of native extinction to the 3/4 reduction level. Enzymatic activity, on the other hand, more nearly follows the course of the alpha/beta ratio. At the half-reduced level, the protein exhibits 2-5% of the specific activity of the native enzyme, while at the fully reduced level it is non-existent. It is not yet possible to differentiate between the possible contamination of the half-reduced protein with active material, and a possible inherent activity at this level.

Earlier (Project No. Z01 HL 00405-05 LCB), it was reported that methanol influences reduced lysozyme to assume a CD behavior interpreted by other workers as indicating a return to native structure. Curve fitting of the CD data, however, had shown this structure not to resemble that of the native protein. It would be crucial to know, for the theory of chain folding, if the "nucleative" forces within the fully reduced chain would suffice for folding to native secondary structure in the absence of SS formation.

In a continuation of this effort, partially and fully reduced lysozyme samples in various concentrations of methanol up to 20% (V/v) were chromatographed on columns of Bio-Gel P-60, which can differentiate between molecular weights under 60,000. From all such experiments, only two peaks have been obtained: a monomer, and a polymer, with the latter moving near the front and thus representing a molecular weight of ≥60,000. With increasing methanol concentration, the polymer peak increased in size while the monomer decreased.

The critical observation from these experiments is that the CD behavior of the monomer proved to be constant regardless of methanol concentration and closely matched that obtained for the same protein in the absence of methanol. Therefore, methanol did not change CD behavior of the monomer, a further indication of the inability of the denatured chain to "nucleate" in the absence of SS formation. It is further significant that these results are contrary to the widely accepted principle that organic solvents have profound effects on polypeptide secondary structure. Thus, many reported observations to that effect may have been the result of polymer formation, rather than changes in secondary structure.

Significance to bio-medical research: This effort is aimed at further elucidation of the mechanism of chain folding. Such information may contribute to understanding better the relationship between primary structure and protein conformation, and may ultimately make possible the reliable prediction of native conformation from knowledge of amino acid sequence. This capability would be basic to the attack of many problems relating to the biological functions of proteins occurring in various conformations.

Proposed course of project: Further experiments are contemplated, to determine the relationship between specific SS bonds and secondary structure.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00409-10 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Interaction of Actin and Myosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Evan Eisenberg	Section Head, Cell Physiology	LCB, NHLBI
Others:	Leonard Stein	Guest Worker	LCB, NHLBI
	P. Boon Chock		LB, NHLBI

COOPERATING UNITS (if any)

Laboratory of Biochemistry, NHLBI

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.3	1.3	--

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Steady-state and pre-steady-state kinetic studies on the interaction of actin, myosin-subfragment-one (S-1) and ATP were carried out to better elucidate the mechanism of the actin activated myosin ATPase. In particular, we tested whether there is mandatory detachment of myosin from actin during each cycle of ATP hydrolysis as proposed by the Lymn-Taylor model. Our studies on the rate and magnitude of the ATP hydrolysis step (initial Pi burst) in the presence of actin are in strong contradiction to the predictions of the Lymn-Taylor model that this step only occurs when the myosin-ATP is detached from actin. We find that the Lymn-Taylor model cannot simultaneously fit the steady state and pre-steady-state kinetic data. Therefore our results show that myosin does not detach from actin during each cycle of ATP hydrolysis; the ATP hydrolysis step occurs at an even faster rate when the myosin ATP is bound to actin than when it is dissociated from actin.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: The accepted mechanism for the contraction of muscle in vivo involves the interaction of actin and myosin filaments. However, the exact nature of the energy transduction mechanism by which the energy stored in the ATP molecule (relative to ADP and Pi) is converted to useful work is not well understood. By studying the biochemical interaction of actin and myosin in vitro we hope to gain insight into the in vivo energy transduction mechanism.

Last year, we proposed a new model for the actin-activated myosin subfragment-1 (S-1) ATPase activity. This model was based on pre-steady-state and steady-state studies of the binding of S-1 to actin in the presence of ATP, and the fact that no inhibition of the actin-activated ATPase by actin had ever been observed:

$$\begin{tikzcd}
\mathrm{M} \arrow[r, shift left] \arrow[d, shift right] & \mathrm{M{\cdot}ATP} \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & \mathrm{M{\cdot}ADP{\cdot}Pi_R} \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & \mathrm{M{\cdot}ADP{\cdot}Pi_N} \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & \mathrm{M{\cdot}ADP} \arrow[l, shift left] \arrow[r, shift left] \arrow[d, shift right] & \mathrm{M} \arrow[l, shift left] \arrow[d, shift left] \\
\mathrm{A{\cdot}M} \arrow[r, shift left] \arrow[u, shift right] & \mathrm{A{\cdot}M{\cdot}ATP} \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & \mathrm{A{\cdot}M{\cdot}ADP{\cdot}Pi_R} \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & \mathrm{A{\cdot}M{\cdot}ADP{\cdot}Pi_N} \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & \mathrm{A{\cdot}M{\cdot}ADP} \arrow[l, shift left] \arrow[r, shift left] \arrow[u, shift right] & \mathrm{A{\cdot}M} \arrow[l, shift left] \arrow[u, shift left]
\end{tikzcd}$$

where M = myosin and A = actin. In this model, instead of the S-1 molecule cycling between states bound to and dissociated from actin, the S-1 cycles between states weakly bound to actin ($\mathrm{M{\cdot}ATP}$, $\mathrm{M{\cdot}ADP{\cdot}Pi_R}$ and $\mathrm{M{\cdot}ADP{\cdot}Pi_N}$) and states strongly bound to actin ($\mathrm{M{\cdot}ADP}$ and M). As in the original refractory state model there is a special rate-limiting step ($\mathrm{M{\cdot}ADP{\cdot}Pi_R \longrightarrow M{\cdot}ADP{\cdot}Pi_N}$) but in contrast to the previous model, this step can occur with the S-1 either bound to or dissociated from actin.

During the past year, we have tested a key feature of this model: that there is no mandatory detachment step, i.e., that it is not required for myosin to detach from actin during each cycle of ATP hydrolysis. The most direct method of determining whether a mandatory detachment step occurs during the ATPase cycle is to study the effect of actin on the initial Pi burst, itself, since this is the step in which the Lymn-Taylor model occurs only with the S-1 dissociated from actin. Therefore in the present study we tested the effect of actin on both the rate and magnitude of the initial Pi burst.

Methods and Findings: Because, in the Lymn-Taylor model, the initial Pi burst only occurs with the S-1 dissociated from actin, mathematical modelling shows that this model makes very different predictions for the rate and magnitude of the initial Pi burst than does our model. The Lymn-Taylor model predicts that, as the actin concentration is increased, the rate of the initial Pi burst will either decrease or remain nearly constant. It also predicts that the magnitude of the initial Pi burst will markedly decrease at high actin concentration. We tested these predictions by using fluorescence enhancement to measure the rate of the initial Pi burst and by using direct assay of Pi to measure the magnitude of the initial Pi burst. We also used direct assay of Pi to determine the steady-state ATPase rate at very high actin concentrations where the Lymn-Taylor model predicts marked inhibition should occur. In all cases our results were inconsistent with the predictions of the Lymn-Taylor model. At high actin concentrations the rate of the initial Pi burst increased markedly, the magnitude of the initial Pi burst decreased only slightly and there was no inhibition of the steady-state ATPase activity. These results were not consistent with the Lymn-Taylor model but they could be accounted for by our kinetic model for the actomyosin ATPase. We therefore conclude that the ATP hydrolysis step can occur with the S-1 bound to actin; there is no mandatory dissociated of the actin and myosin during each cycle of ATP hydrolysis. This work is presently being prepared for publication.

Proposed Course of Project: Having shown that the ATP hydrolysis step occurs with the S-1 bound to actin, we now plan to provide further evidence for the second major feature of our kinetic model -- that the rate-limiting step occurs with the S-1 both unattached and attached to actin. The key manifestation of this feature of our model is that the binding constant of M·ATP and M·ADP·Pi to actin is considerably weaker than the K_{app} for the ATPase activity. Using the purified isoenzymes of S-1, A-1-S-1, and A-2-S-1 which differ in their K_{app}, we will thorougly investigate the ionic strength and temperature dependence of K_{app} and the binding constant of M·ATP and M·ADP·Pi to actin. In this way we should be able to conclusively demonstrate that our kinetic model is the simplest model which can account for the acto·S-1 ATPase activity.

Publications

Stein, L.A., Schwarz, R.P., Chock, P.B., and Eisenberg, E.: Mechanism of actomyosin adenosine triphosphatase. Evidence that adenosine 5'-triphosphate hydrolysis can occur without dissociation of the actomyosin complex. Biochemistry 18:3895-3909, 1979.

Adelstein, R. S., and Eisenberg, E.: Regulation and kinetics of the actin myosin-ATP interaction. Ann. Rev. Biochem. 49: 921-956, 1980

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00411-05 LCB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Respiration-linked H^+ ejection by reconstituted E. coli succinoxidase vesicles.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard W. Hendler	Section Head	LCB NHLBI
Other:	Oruganti H. Setty	Visiting Fellow	LCB NHLBI
	Richard I. Shrager	Mathematician	LSMM DCRT

COOPERATING UNITS (if any)

Laboratory of Statistical and Mathematical Methodology, DCRT

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Membrane Enzymology

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A very sensitive and stable ΔpH meter was developed allowing the measurement of ΔpH in the range of a few ten-thousandths of a pH unit per minute. With this meter we have learned that succinoxidase vesicles prepared by a variety of techniques are capable of net proton ejection with about 10% the H^+/O ratio as intact membranes. The principal reasons why the measured ratio is not higher appear to include an impairment in substrate transport, membranes that are leaky to protons, and extent of true asymmetric reconstitution. Attempts are underway to obtain reconstituted membranes with maximal H^+/O ratios.

PHS-6040
(Rev. 10-76)

Major findings: The current studies are the first to show that succinoxidase, a complex multi-enzyme network requiring strict spatial orientation in a membrane, can be reconstituted from soluble components with energy transducing capability. However, the net number of protons ejected per atom of oxygen reduced is only about 10% of that seen in the native membrane. Because of this, the change in millivolts of the pH signal approached the noise level of the highly sophisticated commercial volt meter used in these studies, introducing serious uncertainty in the quantitative measurements. In order to proceed further with this problem, it became necessary to develop an extremely sensitive and stable new kind of ΔpH meter. This was eventually accomplished in collaboration with Walter Friauf, Chief of the Electrical and Electronic Engineering Section, BEIB.

This new meter works on the principle of null point rejection and employs a pair of matched and extremely quiet amplifiers. A heavy metal cable shield picks up extraneous signals. This shield signal is fed into both amplifiers plus the pH signal in one and the reference signal in the other. This plus other changes in the system and immediate environment allows the measurement of changes of a few ten-thousandths of a pH unit per minute to be made reproducibly and with confidence. With this new instrumentation several different techniques for reconstitution were evaluated. These included gel filtration, Dowex 1, BioRAD SM_2 Biobeads, and dialysis. The highest extent of proton ejection was obtained with the dialysis procedure; Gel filtration and the Biobeads gave results with lower efficiency and the Dowex procedure depressed succinoxidase activity. Proton ejection of vesicles formed by gel filtration could be enhanced by pre-incubating with succinate, indicating that succinate transport may be a limiting factor in the response of the reconstituted vesicles. However, the most serious limitation in obtaining high ratios for H^+ ejection to O_2 reduction is that the reconstituted membranes are leaky to protons and are thus uncoupled. A series of important controls was performed to show that the reconstituted membranes are truly capable of transducing a portion of the succinoxidase respiratory energy into an electrochemical potential. For example, when succinoxidase activity is suppressed by four different techniques, namely (1) replacing oxygen with nitrogen; (2) adding malonate; (3) removing substrate; (4) adding cyanide, no proton ejection occurs. On the other hand, when very high oxidase activity is elicited by D-lactate in place of succinate, no proton ejection is seen.

Proposed course of project: Other techniques for reconstitution will be tried in order to obtain higher H^+/O ratios. The vesicles obtained by reconstitution will be fractionated in order to see if a population of high efficiency vesicles exist in a mixture with inactive vesicles.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00413-04 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Binding of the Subfragments of Myosin to Actin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lois Greene	Staff Fellow	LCB, NHLBI
Other:	Evan Eisenberg	Section Head, Cellular Physiology	LCB, NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: .95	PROFESSIONAL: .95	OTHER: --

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The binding of myosin subfragment-one (S-1) to the F-actin-troponin-tropomyosin complex (regulated F-actin) was examined in the presence of ADP at μ=0.22M, 22°C. S-1·ADP binds with positive cooperativity to regulated F-actin, both in the presence and absence of calcium, while it binds independently to unregulated actin. With and without Ca^{2+} at very low levels of occupancy of the regulated actin by S-1·ADP, S-1·ADP binds about 3-fold more strongly to the regulated actin than it does to unregulated actin. The major difference between the results obtained in the presence and absence of Ca^{2+} with regulated actin is that in the absence of Ca^{2+}, the binding of S-1·ADP remains weak until a higher free S-1·ADP concentration is reached and the transition to strong binding is much more cooperative. These results are consistent with a cooperative binding model where the regulated actin filament can exist in two forms, a weak binding and a strong binding form with Ca^{2+} and S-1·ADP as allosteric effectors shifting the equilibrium between the two forms. These studies also show that troponin-tropomyosin has the ability to greatly weaken the binding of S-1·ADP to actin, both in the presence and absence of Ca^{2+}, although as shown in the accompanying annual report (00417-01) the troponin-tropomyosin has almost no effect on the binding of S-1·ATP to actin.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: In skeletal muscle, the regulation of muscle contraction appears to be controlled by the troponin-tropomyosin complex which binds to the actin filament to form regulated F-actin. The most widely accepted mechanism of troponin-tropomyosin actin is the steric blocking model of Huxley and Haselgrove which suggests that the position of the tropomyosin molecule on the actin filament controls the actin-myosin interaction. Since the tropomyosin molecule spans a unit of seven F-actin monomers, the position of the tropomyosin is thought to be effective over the entire actin unit and, therefore, this model suggests that cooperativity is an inherent part of regulation.

On a biochemical level, cooperative responses of the ATPase activity of regulated acto·S-1 have previously been observed both in the presence and absence of calcium. However, equilibrium binding studies of S-1 to regulated actin have not previously been carried out. Since the cooperative responses observed with regulated acto·S-1 are fundamental to our understanding of the biochemical basis of regulation, the binding of S-1 to regulated actin was examined under equilibrium conditions both in the presence and absence of calcium.

Methods Employed and Major Findings: The binding of S-1 to regulated F-actin was examined under varying conditions both in the presence and absence of calcium. These studies were performed by mixing varying concentrations of S-1 blocked at SH_1 with $[^{14}C]$-iodoacetamide with regulated F-actin. After centrifuging the acto·S-1 complex, the concentration of S-1 which remained in the supernatant was measured to determine the extent of binding.

In the presence of ADP at μ=0.22M, 22°, S-1·ADP binds with positive cooperativity to regulated F-actin both in the presence and absence of calcium, while it binds independently to unregulated F-actin. With and without calcium at very low levels of occupancy of the regulated actin by S-1·ADP, S-1·ADP binds over 100-fold more weakly to the regulated actin than it does to unregulated actin, whereas at high levels of occupancy, S-1·ADP binds about 3-fold more strongly to the regulated actin than to unregulated actin. These results are consistent with a typical cooperative binding model where the regulated actin filament can exist in two forms, a weak binding and a strong binding form. Calcium and S-1·ADP, acting as allosteric effectors, shift the equilibrium toward the strong form.

The binding of S-1 to regulated actin was also examined at low ionic strength, conditions where the ATPase activity of regulated acto S-1 is studied. These experiments were done in the presence of AMP-PNP to obtain detectable dissociation of the acto·S-1 complex. Similar to the results obtained at higher ionic strength, the binding of S-1·AMP-PNP to regulated actin at μ=0.05M, 22°, showed cooperativity both in the presence and absence of calcium. Cooperative binding of S-1 alone to regulated actin has been observed in the absence of calcium at high ionic strength.

These studies show that troponin-tropomyosin has the ability to greatly weaken the binding of S-1·ADP and S-1 to actin, both in the presence and absence of Ca^{2+}, although, as shown in the accompanying annual report, the troponin-tropomyosin has almost no effect on the binding of S-1·ATP to actin. Inhibition of S-1·ADP binding to actin could be related to blocking the release of Pi from the S-1·ADP·Pi complex, i.e., to the formation of S-1·ADP from S-1·ADP·Pi. As pointed out in the accompanying annual report, inhibition of Pi release could be the mechanism by which troponin-tropomyosin inhibits the acto·S-1 ATPase in the absence of Ca^{2+}. The differential effect of troponin-tropomyosin on S-1·ATP and S-1·ADP binding also supports the view that S-1·ATP and S-1·ADP bind in different ways, i.e., perhaps at different angles, to actin.

Significance to Biomedical Research: The interaction of myosin with regulated actin both in the presence and absence of calcium is fundamental to understanding the molecular basis of regulation. This research helps to elucidate the relationship between the cooperative response and the mechanism of relaxation.

Proposed Course of Research: The plan for this project is to continue examining the binding of S-1 to regulated actin. First, to test the cooperative model we have developed for this system in cooperation with Dr. Terrell Hill (see accompanying annual report), the effect of different nucleotides on the cooperative response will be examined. This is a test of our model since the model predicts that the cooperative response will be independent of nucleotide. The effect of calcium concentration on the binding of S-1 to regulated actin will also be examined to see if the results fit the predictions of our model. To determine their role in the cooperative response, the effect of pure tropomyosin, tropomyosin cleaved at its ends (so adjacent tropomyosin molecules cannot interact), and the individual troponin components on the binding of S-1 to regulated actin will be determined. In addition, the binding of heavy meromyosin to regulated actin will be examined to determine the cooperative response which occurs with this two-headed fragment of myosin.

Another aspect of this project is to determine the relationship between the weak form of actin and the ATPase activity of regulated acto·S-1, i.e., to determine whether the weak form alone, either in the presence or absence of calcium, is sufficient to inhibit the ATPase activity. This will be done in collaboration with Dr. Joseph Chalovich where the proportion of actin in the weak form in calcium will be correlated with the actin activation of regulated acto·S-1 in the presence of calcium. In addition, ATPase activities of acto·S-1 and binding studies will be conducted in the presence of tropomyosin using *Acanthamoeba* actin. Since there is no inhibition of ATPase activity with *Acanthamoeba* actin in the presence of tropomyosin, it is important to establish whether S-1 binds cooperatively to the tropomyosin-*Acanthamoeba* actin complex.

Publications

Greene, L.E., and Eisenberg, E. The binding of heavy meromyosin to F-actin. *J. Biol. Chem.* 255:549-554, 1980.

Greene, L.E. and Eisenberg, E. Cooperative binding of myosin subfragment-one to the actin-troponin-tropomyosin complex. Proc. Natl. Acad. Sci. USA 77:2616-2620.

Greene, L.E., and Eisenberg, E. Dissociation of the actin·subfragment-one complex by adenyl-5'-yl imidodiphosphate, ADP, and PP_i. J. Biol. Chem. 255 #2: 543-548, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00415-02 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Models of Muscle Contraction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Evan Eisenberg	Section Head, Cellular Physiology	LCB,NHLBI
Others:	Lois Greene	Staff Fellow	LCB,NHLBI
	Terrell Hill	Section Head, TMB	LMB,NIAMDD

COOPERATING UNITS (if any)

Theoretical Molecular Biology, Laboratory of Molecular Biology, NIAMDD

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

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.50	.50	--

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Modelling of several aspects of muscle contraction is presently underway. First, we are quantitatively modelling our new cross-bridge model which is based on the concept that the myosin cross-bridge oscillates between states weakly bound to actin with a preferred angle of about 90° and states strongly bound to actin with a preferred angle of about 45°. The former states have bound ATP or ADP + Pi while the latter states have no bound Pi. The concept of two major types of cross-bridge states is combined with the biochemical concept of rapid equilibria between attached and detached states and the physiological concept of an elastic cross-bridge to allow a model in which the myosin does not detach from actin during each cycle of cross-bridge action but does mechanically detach when the muscle is shortening. In addition to modelling cross-bridge actin in vivo, we are currently modelling the cooperative actions of the troponin-tropomyosin-actin complex which we have observed in our experimental work. This involves detailed modelling of the cooperative binding of myosin·ADP to the troponin-tropomyosin-actin complex, a phenomenon which we have observed in our experimental work. It also involves modelling the cooperative effects of troponin-tropomyosin on the steady-state actin-activated myosin ATPase activity.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: It is now generally recognized that contraction of muscle involves the interaction of the two proteins, actin and myosin, with ATP. Based on our experimental work, both with ATP and with ATP analogues, we have developed a kinetic model for the actomyosin ATPase which has several distinguishing features. First, the binding of ATP does not irreversibly dissociate the actomyosin complex during each cycle of ATP hydrolysis; myosin-ATP is in rapid equilibrium with actomyosin-ATP. Second, ATP hydrolysis can occur without dissociation of the actomyosin complex. In fact, the ATP hydrolysis step may be even faster when the myosin is attached to actin than when it is dissociated from actin. Third, the slowest step in the cycle is the transition from the refractory to the non-refractory state which precedes the rapid release of Pi and can occur with the myosin either bound to or dissociated from actin. The theoretical formalism of T.L. Hill provides a method of relating this kinetic model to cross-bridge behavior in vivo. We have previously published a qualitative model of cross-bridge action based on this kinetic model and are presently developing a quantitative version of this in vivo cross-bridge model.

We are also modelling the cooperative action of the troponin-tropomyosin complex in vitro. This modelling is directly related to our experimental observations on the cooperative body of S-1, and its various nucleotide complexes to regulated actin filaments.

Methods Employed and Major Findings: The cross-bridge model which we are presently quantitatively modelling has the following major features. First, and most important, the myosin cross-bridge does not undergo a simple attachment-detachment cycle in vivo. Rather, the cross-bridge cycles between states weakly attached to actin at about 90° and states strongly attached to actin at about 45°. Since there is no mandatory detachment step, the cross-bridge only detaches when it is elastically distorted and is thus in rapid equilibrium with an attached state. Second, the rapid release of Pi is associated with the transition from the 90° to the 45° state and the rapid recovery of force in the isomeric transient. Third, the rate-limiting transition from the refractory to the non-refractory state controls the shortening velocity by limiting the number of bridges which make the transition from the 90° to the 45° state and thus go through a complete force-producing cycle.

One of the important features of our quantitative modelling is that, for the first time, we are including almost all of the biochemical states observed in vitro. Therefore, in this new model there is a quantitative correspondence between the relative equilibrium levels of biochemical states in vitro and their relative free energy in vivo. At the present time we are quantitatively modelling the rapid isometric transient and will shortly begin modelling the steady-state force-velocity curve.

Turning to our modelling of the cooperative effects of troponin-tropomyosin, our first effort was devoted to modelling our experimental data on the equilibrium binding of S-1 to regulated actin filaments both in the presence and in the absence of Ca^{++}. In our model, each tropomyosin-troponin unit (including seven sites on the actin filament) can be in one of two possible states, which have different intrinsic free energies and different binding constants for S-1.

Bound S-1 molecules do not interact with each other. There are nearest-neighbor (pair) interactions between these units that depend on the state of each member of the pair and on the number of Ca^{++} ions bound to one member of the pair. There are two sources of positive cooperativity in this system: the fact that seven actin sites change state together as part of a single unit; and the existence of attractive nearest-neighbor interactions between units. Parameters in the model are evaluated by fitting the data, both in the presence and in the absence of Ca^{++}. A description of this model is in press at PNAS.

In addition to this theoretical work on the cooperative equilibrium binding of S-1 to regulated actin, as influenced by Ca^{++}, we have extended our modelling efforts to the cooperative steady-state ATPase activity of myosin subfragment-1 on regulated actin. Exact solution of the general steady-state problem requires Monte Carlo calculations. However, using approximations, we have examined three interrelated special cases and in a paper submitted to Biophys. J. sample computer (not Monte Carlo) solutions are given. The eventual objective of this modelling effort is to apply our model to *in vitro* experimental data and to *in vivo* muscle models.

Proposed Course of Project: We will continue the quantitative modelling of our new cross-bridge model with emphasis on modelling the force-velocity curve. In addition, we will continue our modelling of the cooperative actions of the troponin-tropomyosin complex. In particular, we will attempt to develop a model for relaxation *in vivo*.

Publications:

Eisenberg, E. and Greene, L.E: The relation of muscle biochemistry to muscle physiology. Annual Review of Physiology 42: 293-309, 1980.

Eisenberg, E., Hill, T.L., and Chen, Y.-D.: A cross-bridge model of muscle contraction: quantitative analysis. Biophys. J. 29:195, 1980.

Hill, T.L., Eisenberg, E., and Greene, L.E.: A theoretical model for the cooperative equilibrium of myosin subfragment-one to the actin-troponin-tropomyosin complex. Proc. Natl. Acad. Sci. USA 77:3186-3190, 1980.

Hill, T.L. and Eisenberg, E. Theoretical considerations in the equilibrium binding of myosin fragments on f-actin. Biophys. Chem. 11:271-281, 1980.

251

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00416-01 LCB

PERIOD COVERED
October 1, 1979-September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparative studies on plasma proteins from normal and cystic fibrosis patients

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Frederick H. White, Jr.	Senior Investigator	LCB NHLBI
Other:	P. A. Di Sant´Agnese	Chief	PM NIAMDD

COOPERATING UNITS (if any)

Pediatric Metabolism Branch, NIAMDD

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The basic genetic defect responsible for cystic fibrosis (CF) is as yet unknown, and its elaboration should ultimately result in improving the treatment of this disease. There have been reports that α_2 macroglobulin (α_2M) from plasma, which is known to inhibit protease activity, is less potent as an inhibitor in CF patients. This difference suggests a structural variation that might be the result of a genetic defect. These reports have, however, been controversial, and the work thus far in this laboratory has centered on confirming and extending the reports on α_2M. This protein may be digested with trypsin. Early indications have suggested a difference in the rate of digestion, that of the CF α_2M being slower.

PHS-6040
(Rev. 10-76)

Project Description

Objective: To isolate and examine plasma proteins from normal and cystic fibrosis (CF) individuals, in a search for structural differences related to the genetic defect of CF.

Methods employed:
(1) Well known methods of separation and purification of plasma proteins, involving ammonium sulfate fractionation, ion exchange chromatography and gel filtration chromatography have been used in purification of α_2-macroglobulin (α_2M) from human plasma.
(2) Various methods are being employed (or are being contemplated) for the characterization of isolated α_2M. These include colorimetric and titrimetric trypsin assays (to determine the extent to which α_2M inhibits proteolytic activity), amino acid analysis on a Beckman Model 120 automatic amino acid analyzer, gel filtration chromatography (with Bio-Gel resins) to separate components after tryptic digestion, immuno-diffusion studies, and analyses to determine the nature of the carbohydrate content of α_2M (by the methods of Ben-Yosef *et al*., Clin. Chim. Acta 99, 31 (1979)).

Major findings: Isolated samples of α_2M from normal and CF individuals have been reported (Shapiro *et al*., J. Biol. Chem. 252, 7923, (1977)), to show differences in their ability to inhibit proteases. Work in this laboratory has centered around efforts at repeating these observations, since they appear controversial, and may be crucial for pinpointing the genetic defect of CF.

Thus far, we have not confirmed the decreased inhibition of trypsin by α_2M from CF patients. There appears, however, to be a difference in the rate at which α_2M is digested by trypsin. Thus, gel filtration chromatography of the digest shows less breakdown of the CF α_2M than of the normal α_2M.

Significance to biomedical research: The immediate goal of the present effort is to resolve the disputed claims that normal and CF α_2M behave differently in several respects. This work more generally is aimed at understanding the genetic defect of CF.

Proposed course: We will investigate further the possibility of structural differences in normal and CF α_2M, and, if such differences are found, examine them in detail.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00417-01 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Mechanism of Regulation of Acto-S-1 ATPase by Troponin-Tropomyosin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Evan Eisenberg	Section Head, Cellular Physiology	LCB, NHLBI
Other:	Joseph Chalovich	Staff Fellow	LCB, NHLBI

COOPERATING UNITS (if any)

none

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Cellular Physiology

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.00	1.00	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The steric blocking model of troponin-tropomyosin action suggests that relaxation of skeletal muscle is caused by tropomyosin physically preventing the binding of the myosin cross-bridge to actin in the absence of Ca^{2+}. We tested this model by measuring the binding of myosin subfragment-one (S-1) to actin and regulated actin (actin + troponin + tropomyosin) with and without Ca^{2+} in the presence of ATP. Stopped-flow turbidity measurements were used to determine the amount of S-1 binding to actin. At 25°C, μ=17.5mM the binding constant of S-1 to actin or regulated actin in the presence of Ca^{2+} is 2.3 x $10^4 M^{-1}$. In the absence of Ca^{2+}, when the ATPase is 94% inhibited, the binding of S-1 to regulated actin is reduced only two-fold (K=1.3 x $10^4 M^{-1}$). These data show that the inhibitory effect of troponin-tropomyosin on the actin activated S-1 ATPase without Ca^{2+} is not due to inhibition of binding of S-1 to the actin filament in the presence of ATP. Rather, these data suggest that troponin-tropomyosin acts by blocking the release of Pi from the actin-S-1-ADP-Pi complex. In vivo this may correspond to blocking the rotation of the actin bound cross-bridge from the 90° attached state to the 45° attached state.

Project Description

Objectives: The purpose of this work is to understand the mechanism by which the troponin-tropomyosin complex confers Ca^{2+} sensitivity on the actin activation of the S-1 ATPase. The first question to be answered is whether the troponin-tropomyosin complex inhibits the actin activation of the S-1 ATPase, in the absence of Ca^{2+}, by decreasing the association of S-1 with actin in the presence of ATP. The second goal is to investigate the changes in the actin-S-1 ATPase activity which are caused by troponin-tropomyosin.

Methods Employed and Major Findings: Binding of S-1 to actin or regulated actin, in the presence of ATP, was measured from turbidity observed in a stopped-flow spectrophotometer. ATPase rates were simultaneously estimated from the time course of turbidity changes which accompanied ATP hydrolysis. It was concluded that, at 25°C, μ=17.5mM, the troponin-tropomyosin complex conferred Ca^{2+} sensitivity on the ATPase rate but not on the binding of S-1·ATP to actin. In the absence of Ca^{2+} the ATPase was only 6% of the rate with Ca^{2+} although the binding constant of S-1 to actin was weakened only 2-fold. These data suggest that troponin-tropomyosin acts by preventing Pi release from the actin-S-1·ADP·Pi complex rather than by blocking the binding of S-1·ATP to actin.

The effect of troponin-tropomyosin on the acto-S-1 ATPase was also investigated as a function of S-1 and actin concentration. In the absence of Ca^{2+} and presence of regulatory proteins the rate does not increase with increasing actin concentration after an initial 4-fold increase in the rate above that of S-1 alone. In the presence of Ca^{2+} a linear double reciprocal plot of ATPase vs actin is obtained. The maximum ATPase rate is roughly 300-fold greater than that of S-1 in the absence of actin. This is almost the same as the maximum ATPase rate in the absence of regulatory proteins. However, the K_{app} for actin is one-third the value observed in the absence of regulatory proteins. At very high S-1 concentration cooperative effects are observed in the presence of regulatory proteins. These cooperative effects are presently being investigated as is the effect of regulatory proteins on K_{app}.

Significance to Biomedical Research: Understanding the mechanism by which the troponin-tropomyosin complex confers Ca^{2+} sensitivity on the actin activated S-1 ATPase activity is important in understanding both skeletal muscle contraction and cell motility in general. This knowledge may have particular application in the study of various muscular dystrophies of unknown etiology as well as in the study of heart disease.

Proposed Course of Research: The demonstration that S-1 binds to regulated actin in the absence of Ca^{2+} and presence of ATP, although the ATPase activity is inhibited, will be confirmed. Studies will be undertaken which directly measure the amount of S-1-ATP bound to actin by an ultracentrifugation method. A kinetic method will also be employed at varying Ca^{2+} concentrations to show that S-1•ATP is always bound to regulated actin.

The kinetic studies which have been started will be extended to higher con-

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centrations of actin and S-1 using a radioactive ATPase assay. The effect of the regulatory proteins on the steady-state actin-activated S-1 ATPase rate will be investigated at a series of temperatures. The effect of regulatory proteins on the pre-steady-state hydrolysis of ATP (initial Pi burst) will also be investigated.

Publications

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00501-06 LCB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Actin of Nonmuscle Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward D. Korn	Chief	LCB, NHLBI
Other:	Stephen Mockrin	Staff Fellow	LCB, NHLBI
	Stephen Brenner	Staff Investigator	PSL, DCRT

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cytochalasin D inhibits the polymerization of actin, raises the critical concentration of monomeric actin, and greatly increases the ATPase activity of actin. When added to polymerized actin, cytochalasin D causes rapid depolymerization to a new steady state level. Spectrin/actin complex from red blood cells nucleates and accelerates the polymerization of actin and lowers the critical concentration of monomeric actin at steady state. Cytochalasin D inhibits polymerization of actin nucleated by spectrin/actin complex but does not depolymerize the actin. These observations support a model of actin polymerization in which the filament elongates at one end more rapidly than at the other. At steady state, actin monomers are continually adding to one end of the filament and leaving the other end. Cytochalasin D, according to this model, would inhibit addition of monomers at the net polymerizing end, perhaps by uncoupling the ATPase reaction, and spectrin/actin complex would block loss of monomers at the net depolymerizing end. These experiments also lead to a picture of the spectrin/actin complex as decameric oligomers of actin crosslinked by spectrin tetramers. In other experiments we have found that profilin forms a weak 1:1 molar complex with actin and enhances the rate of exchange of actin-bound ATP.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Actin is one of the major proteins of the cytoskeleton of all eukaryotic cells. As such, it is involved in many different motile processes and in the regulation of cell shape and cell organization. Actin is a globular molecule of molecular weight 42,000 that polymerizes into double helical filaments under ionic conditions similar to those of the cytoplasm of cells. It is in this polymerized form (microfilaments) that actin presumably functions in non-muscle cells just as it is the polymerized form of actin (thin filaments) that function in muscle contraction. However, in contrast to the situation in muscle, most of the actin in non-muscle cells is non-polymerized and its polymerization is both temporally and spatially regulated so that the microfilaments occur in the cell when and where they are needed for specific motile events. We have described in past years two ways in which the polymerization of actin in non-muscle cells might be regulated. One is through interaction of monomeric actin with profilin, a 14,000-dalton protein that forms a 1:1 complex with actin and inhibits its polymerization. The second mechanism is the interaction of non-polymerized actin with complexes that facilitate the polymerization of actin. An intriguing model for such a nucleating complex is the complex of spectrin and actin that can be isolated from red blood cells. This year, we have attempted to study the nature of the interaction of profilin and actin by the use of a number of specific probes to determine the mechanism by which the spectrin/actin complex facilitates the polymerization of actin and to examine some of the fundamental events of actin polymerization that naturally occurring modulators might affect.

Methods employed and major findings: (1) Interaction of profilin and actin: A number of attempts to link actin and profilin covalently by reaction of the complex with bifunctional reagents were unsuccessful. Similarly, attempts to detect changes in conformation of either actin or profilin in the complex were unsuccessful. Methods used included monitoring the fluorescence of tryptophan residues and of fluorescent probes covalently bound to either protein, and seeking changes in the electron spin resonance signal of appropriately labeled actin upon its interaction with profilin. We were able, however, to observe the interaction of profilin and actin by its effect on the rate of exchange of actin-bound ATP.

Profilin was isolated from Acanthamoeba castellanii as an essentially homogeneous protein by methods described in last year's report. Actin is isolated from either muscle or Acanthamoeba by standard procedures. Monomeric G-actin normally contains one mole of bound ATP per mole and the ATP is exchangeable with ATP in solution. The rate of exchange is measured by first equilibrating G-actin with [^{32}P]ATP and removing all free [^{32}P]ATP by binding it to Dowex-1 chloride. The G-actin-[^{32}P] is then placed in solution with a large molar excess (100-fold) of non-radioactive ATP which will exchange with the radioactive ATP. Samples are removed at timed intervals, treated with the Dowex-1 to remove the free ATP and the residual actin-bound radioactive ATP measured.

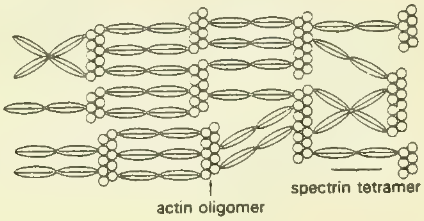
In this way, it was determined that (a) G-actin and the G-actin-profilin complex both contain one mole of bound ATP per mole; (b) in both cases the bound ATP is totally exchangeable with ATP in solution; (c) under the particular conditions used (0.033 mM Ca^{2+}, 0.1 mM ATP, pH 7.5, 25°) the exchange rate for G-actin, $7.7 \times 10^{-4}\ s^{-1}$, was increased 17 times by a 7-fold molar excess of profilin to a value of $1.3 \times 10^{-2}\ s^{-1}$; (d) the binding of profilin to G-actin is rapid and reversible with a K_D of 4.7×10^{-5}M, which is rather weak, and a stoichiometry of 1:1.

These data, therefore, confirm the previous indication for the stoichiometry of the complex and further indicate that profilin greatly weakens the association of ATP for actin. Whether this is of physiological significance is not now known but hydrolysis of actin-bound ATP to actin-bound ADP occurs concomitantly with actin polymerization and the ability of profilin to reduce the affinity of actin for ATP might affect the rate of nucleation of actin.

(2) Nucleation of actin polymerization by spectrin/actin complex: Reports from other laboratories had suggested that erythrocyte spectrin could induce the polymerization of actin under conditions in which actin would not polymerize alone. Last year we showed that this putative property of spectrin was, in fact, attributable not to spectrin but to a complex of spectrin and actin (as isolated from sheep erythrocytes; from human erythrocytes the complex also includes a third protein, band 4.1). This year we have shown that the effect of the complex is to nucleate actin polymerization, i.e. to accelerate the polymerization of actin, and to lower the concentration of actin necessary for polymerization to occur (lower the critical concentration).

The spectrin/actin complex was isolated from washed ghosts of sheep erythrocytes by extraction at very low ionic strength and purified by molecular seive chromatography on agarose. In solutions containing 0.3 mM Mg^{2+}, G-actin polymerizes very slowly, as measured by the increase in viscosity, and this rate of polymerization is greatly accelerated by the addition of a small amount of the spectrin/actin complex. The final extent of polymerization, i.e. the final viscosity at steady state, is essentially the same whether or not polymerization was accelerated by the complex. This is just the behavior observed for nucleation of actin polymerization by short fragments of F-actin and, therefore, the complex behaves as if it consists of short pieces of polymerized actin stabilized by crosslinks of spectrin tetramer. (Experiments reported last year demonstrated that spectrin tetramer does crosslink F-actin in just this way.) Also similar to the behavior of actin nuclei was the ability of spectrin/actin complex to overcome the inhibition of actin polymerization caused by profilin. The spectrin/actin complex, therefore, may be a prototype for natural nucleating centers in cells. But it is equally likely that the properties of the isolated complex are just an inevitable consequence of its structure with little wider significance.

It was known from the work of Dr. Shin Lin at Johns Hopkins University that the spectrin/actin complex accounted for all of one class of high affinity cytochalasin-binding sites in the erythrocyte. From a combination of these data, knowledge of the actin content in the ertythrocyte and evidence discussed below that cytochalsin binds to one end of actin filaments, we could calculate that the spectrin/actin complex probably consists of decamers of polymerized actin cross-linked by spectrin tetramers. The isolated complex is probably just a mechanical fragment of a continuous network in the cell.

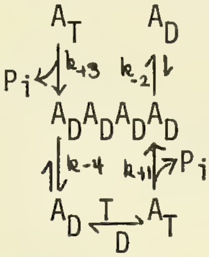


The ratio of one spectrin tetramer for every four actin monomers pictured above is the ratio in which the two molecules occur in the human red blood cell. From the data of others, the spectrin-actin crosslinks are probably stabilized by a third protein, band 4.1, and the cytoskeleton is linked to the integral membrane protein, band 3, by a fourth protein, band 2.1, which binds to spectrin and band 3.

(3) Effect of cytochalasins on actin: The cytochalasins are a group of natural products that are potent inhibitors of a variety of motile processes in eukaryotic cells that are thought to be related to actin function. This year we observed (a) that the cytochalsins greatly inhibited the rate of polymerization of actin; (b) that cytochalasin D caused rapid depolymerization of F-actin; (c) that cytochalasin D did not depolymerize F-actin when its polymerization had been nucleated by the spectrin/actin complex but does stop further elongation; (e) that the critical concentration of actin (the concentration of monomeric actin in equilibrium with polymeric actin at steady state) was increased by cytochalsin D; and (f) that cytochalasins, under appropriate conditions, could accelerate the ATPase activity of polymeric and monomeric actins and disassociate the polymerization of actin from the hydrolysis of ATP.

Considerations of observations (a) through (e) and the previous effects of spectrin/actin complex on actin polymerization have provided strong experimental support for a novel model of actin polymerization first proposed on theoretical grounds by a German scientist, A. Wegner. According to previous theory, actin polymerization was viewed as a three step process (1) a rate-limiting nucleation step in which 2 or 3 actin monomers joined, (2) a rapid elongation step in which monomers of actin added to the end of the growing filament, (3) a re-distribution step in which the distribution of filament lengths became less disperse with no additional actin undergoing polymerization. The steady state situation was envisaged as as simple equilibrium between monomer and polymer: $A_n + A = A_{n+1}$ with the concentration of actin monomer $[A]$ at equilibrium being simply the reciprocal of the equilibrium constant: $K_{eq} = [A_{n+1}]/[A_n][A]$ because $[A_{n+1}] = [A_n]$.

Wegner pointed out that the steady state situation was more complicated than this because at least four reactions needed to be considered: the irreversible conversion of monomeric actin-ATP to polymeric actin-ADP + P_i, which could occur at both ends of the filament, and the reversible conversion of monomeric actin-ADP to polymeric actin-ADP, which could also occur at both ends of the actin filament. When polymerization occurs in the presence of excess ATP, monomeric actin-ADP would be converted to monomeric actin-ATP.



where A = actin
T = ATP
D = ADP

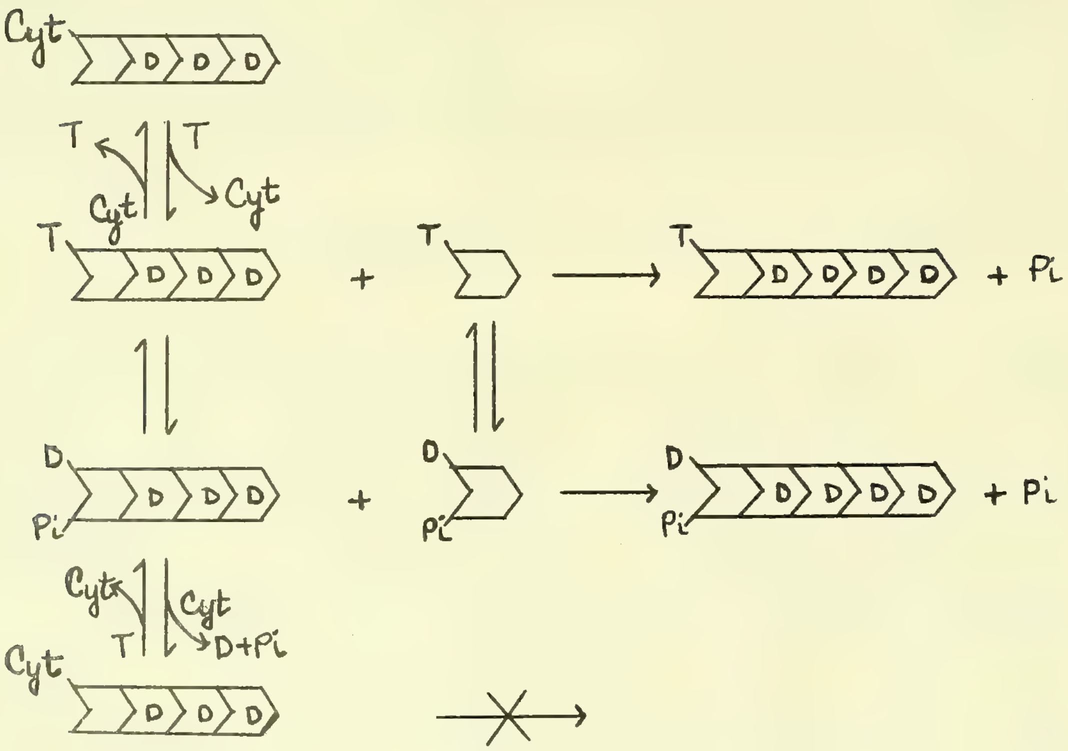
This model and only this model, allows for the possibility that there may be net addition of actin monomers at one end of an actin filament and equal net loss of actin monomers at the other end of the actin filament. Thus, the system as a whole could be at steady state with neither end of the filament being at equilibrium and with actin monomers moving through the filament from one end to the other. The energy for this movement would be provided by the hydrolysis of ATP. According to this concept, the concentration of actin monomer at steady state would not be the reciprocal of an equilibrium constant, there is no equilibrium, but would be some concentration of actin monomer greater than the hypothetical critical concentration at one end and lower than the hypothetical concentration at the other end such that the rate of actin addition at the first end was just equal to the rate of actin loss at the other end.

$$\text{Critical concentration} = [A_T] = \frac{k_{-2} + k_{-4}}{k_{+1} + k_{+3}}$$

Depolymerization of F-actin by added cytochalasin D and increase in the experimentally measured critical concentration of actin in the presence of cytochalasin D could be explained according to this model if cytochalsin D preferentially inhibited events at what would normally be the net polymerizing end of F-actin at steady state (and the preferred addition end during elongation). Actin polymerization would then be envisaged as a process involving nucleation and then elongation at both ends of the growing filament, but probably more rapidly at one end than at the other, until the monomer concentration became too low for elongation to continue at one end. Elongation would continue at the other end but soon the net addition at that end would be balanced by a loss of monomers at the other end.

Additional insight into the polymerization process was obtained when we found that cytochalasin D could also greatly stimulate the hydrolysis of ATP by actin even as it inhibited polymerization so that the normally tight coupling between actin ATPase activity and actin polymerization could be uncoupled. All of these data can be fit to one model, for which there is appreciable additional circumstantial evidence from other laboratories as well.

According to our most recent model for actin polymerization, the ATP that is hydrolyzed to ADP and P_i when an actin filament elongates by one subunit may come from the filament and not from the monomer that is being added; i.e., the actin filament may have a terminal bound ATP. Cytochalasin D may interact with that terminal subunit inhibiting the addition of the next monomeric subunit and also catalyzing the hydrolysis of the filament-bound ATP. Such a reaction would explain the ability of cytochalasin D to stimulate the ATPase activity of actin, to uncouple actin polymerization from actin ATPase, to reduce the rate of actin elongation and to cause the depolymerization (increase the critical concentration) of polymerized actin by inhibiting elongation at the net addition end while leaving unaffected the depolymerization at the net depolymerizing end until the increase in monomeric actin concentration re-established a new steady state at a lower polymer concentration. If, on the other hand, spectrin/actin complex nucleated actin polymerization by blocking the normally net depolymerizing end then this would lead to a lower critical concentration of actin at steady state and also counteract the effect of cytochalasin D when added to polymerized actin by stabilizing the net depolymerizing end of the filament.



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Proposed course of research: A number of data suggest that the effects of cytochalasins A, B, C and E on actin polymerization and actin ATPase activity are not identical to those described for cytochalasin D. These drugs will be examined more closely to see if through their use further insights on the actin polymerization mechanism can be deduced. Suggestive evidence has been obtained that monomeric actin may also interact with cytochalasins (in addition to the now well described interaction of filament ends with the cytochalasins). This possibility will be further investigated. In order to separate events of the nucleation step from the process of actin elongation, cross-linked dimers of actin will be prepared and used to study the elongation reaction and ATPase reactions of growing actin nuclei. The model proposed above implies, as stated, that at steady state there will be a net addition of actin monomers at one end of the filament and net loss at the other end with the filament length remaining constant. Efforts will be made through the use of radioactively labeled actin to obtain direct evidence for this so-called treadmilling of actin and for the extent to which it is tightly coupled to the hydrolysis of ATP.

Publications:

1. Yang, Y.-Z., Korn, E. D., and Eisenberg, E.: Cooperative binding of tropomyosin to muscle and *Acanthamoeba* actin. *J. Biol. Chem.* 254: 7137-7140, 1979.

2. Brenner, S. L., and Korn, E. D.: Spectrin-actin interaction. Phosphorylated and dephosphorylated spectrin tetramer crosslink F-actin. *J. Biol. Chem.* 254: 8620-8627, 1979.

3. Brenner, S. L., and Korn, E. D.: Substoichiometric concentrations of cytochalasin D inhibit actin polymerization: Additional evidence for an F-actin treadmill. *J. Biol. Chem.* 254: 9982-9985, 1979.

4. Brenner, S. L., and Korn, E. D.: The effects of cytochalasins on actin polymerization and actin ATPase provide insights into the mechanism of polymerization. *J. Biol. Chem.* 255: 841-844, 1980.

5. Brenner, S. L., and Korn, E. D.: Spectrin/actin complex isolated from sheep erythrocytes accelerates actin polymerization by simple nucleation: Evidence for oligomeric actin in the erythrocyte cytoskeleton. *J. Biol. Chem.* 255: 1670-1676, 1980.

6. Mockrin, S. C., and Korn, E. D.: *Acanthamoeba* profilin interacts with G-actin to increase the rate of exchange of actin-bound adenosine triphosphate. *Biochemistry* 19: in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00503-08

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure, Assembly and Function of Microtubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin Flavin	Section Head Organelle Biochemistry	LCB/NHLBI
Other:	Nirbhay Kumar	Visiting Fellow	LCB/NHLBI
	Terry Jones	Medical Technologist	LCB/NHLBI
	Jayasree Nath	Visiting Associate	LCB/NHLBI
	Richard Klausner		LTB/NCI
	Elliott Schiffmann		LDBA/NIDR
	John Gallin		LCI/NIAID
	Dan Gilbert		LB/NINCDS
	Ray Lipicky		LB/NINCDS

COOPERATING UNITS (if any) Laboratory of Theoretical Biology, NCI (Project 1b); Laboratory of Developmental Biology and Anomalies, NIDR (Project 4a); Laboratory of Clinical Investigation, NIAID (Project 4b); Laboratory of Biophysics, NINCDS (Project 5)

LAB/BRANCH
Laboratory of Cell Biology

SECTION
Section on Organelle Biochemistry

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD 20205

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4	3	1

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SUMMARY OF WORK (200 words or less - underline keywords)

We are studying a post-translational modification of tubulin involving the reversible enzymatic addition of a C-terminal tyrosine. We have studied whether tyrosylation alters affinity for other cell components with respect to binding of microtubule-associated proteins to microtubules, and of phospholipid vesicles to tubulin. The finding that subunits are preferred by the tyrosylating enzyme, and oligomers or polymers by the detyrosylating enzyme, raises the question of whether the modification is coupled to assembly. In this connection steady-state treadmilling rates have been compared. With respect to tubulin structure, we have found an approach toward characterizing that species which is not susceptible to the modification in vitro, and the fact that this species is modified in vivo has resolved a paradox relating to membrane-bound tubulin. The increased rate of tyrosylation we observed in chemotactically stimulated leukocytes suggests a relation to cytoskeletal rearrangement. Increased rates in leukocytes of patients with Chediak-Higashi disease offer a possible, if remote, chance to find the molecular basis of the disease.

Project Description

Objectives: We are investigating enzymatic reactions by which a tyrosine can be reversibly added to the C-terminus of the α chain of tubulin. Our objective is to discover what this post-translational modification is for.

Methods Employed: Biochemical and cytological procedures as indicated under Major Findings.

Major Findings:

1a. Relative affinities of tyrosylated and detyrosylated tubulin for high molecular weight, assembly-promoting MAP proteins (N. KUMAR): The obvious place to look for a function for this modification is in its effect on interaction between microtubules and subcellular organelles. This binding is probably effected through the microtubule associated (MAP) arm proteins which extend out at 32 nm intervals from the microtubule. We therefore began by comparing the affinity of MAPS for maximally tyrosylated (T-) and completely detyrosylated (D-) tubulin. MAPS isolated from 3 times polymerized (3xp) microtubule protein, by phosphocellulose chromatography, promote tubulin assembly in vitro, and coassemble with tubulin. Native MAPS have never been characterized, but in denaturing gel electrophoresis conditions the fraction shows variable amounts of very faint bands, a distinct 360,000 dalton MAP_1, and predominant amounts of 300,000 dalton MAP_2. A fraction containing almost only the MAP_2, isolated from a Bio-Gel 1.5 m column after boiling 3xp protein, is more effective than the former fraction in promoting assembly.

T- and D-tubulin dimers were isolated by phosphocellulose chromatography after treating 3xp protein with tubulin-tyrosine ligase or carboxypeptidase A (CPA). We have found no differences in rate or extent of assembly between T- and D-tubulin, when various concentrations of the 2 types of tubulin were incubated with various concentrations of either mixed MAPS or MAP_2. Taxol (see 2a), however, appeared to cause faster and more extensive assembly of T-tubulin, as monitored by turbidity. To compare binding affinity more directly, microtubules were spun down from each of the samples with the mixed MAPS, after assembly had reached a plateau. The pellets were analyzed by SDS-PAGE electrophoresis, and the ratio α + β chain/ MAP_1 + MAP_2 was estimated by densitometric scanning of photographic negatives of the Coomassie blue stained gels. The average mass ratios, 5.6 ± 0.33 for 12 T-tubulin samples, and 7.6 ± 0.75 for 12 D-tubulin samples, were significantly different at the 5% level. Whether this reflects different affinities, or numbers of binding sites, could not be established because we could not saturate tubulin with respect to assembly, even with 2:1 (w/w) MAPS to tubulin. Saturation has been reported only with a MAP_2 preparation (Kim et al. JCB 80, 266) and yielded a mass ratio of 3.4.

1b. Tubulin interaction with liposomes (N. KUMAR): Various membrane-bounded vesicles, secretory granules and viruses have been described as appearing to track along microtubules in living cells, or bind to microtubules in vitro. To test whether tyrosylation affects such interaction, we began to study binding of T- and D-tubulin to small, uncharged unilamellar vesicles prepared from dipalmitoyl phosphatidylcholine. The vesicles were prepared containing trapped, water-soluble carboxyfluorescein at 100 mM, a concentration at which fluorescence is almost completely quenched. Because self-

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quenching is relieved by dye leakage, the amount of dye that has leaked out of a population of vesicles can be monitored by the appearance of increased fluorescence. It is this increase in fluorescence that we used to monitor the interaction of tubulin with the vesicles.

Tyrosylation has not so far been found to affect tubulin interaction with liposomes. The interaction has been sufficiently interesting, however, that we have pursued this project from another standpoint, i.e. the nature of brain membrane-bound tubulin, and the possibility that the latter might anchor microtubules to biological membranes.

Tubulin was found to bind strongly to the lipid bilayers at the phase transition temperature; 10-15 tubulin dimers bound per vesicle were sufficient to cause half maximal dye release. Except for serum apolipoproteins, other proteins tested have not shown this property. MAPS did not bind, and MAPS or GTP inhibited tubulin binding. The binding probably involves protein insertion at the phase transition: it was hydrophobic, the vesicles remained intact (apolipoprotein binding destroys the vesicles), and bound tubulin was no longer available to interact with other vesicles. Stable lipid-tubulin complexes were isolated from KBr gradients. Protein conformational changes associated with binding were indicated by circular dichroic spectra, decreased tryptophane fluorescence, and increased KI quenching, although the tubulin remained competent to bind colchicine. Limited proteolysis experiments provided further evidence that the tubulin was actually sequestered in the lipid bilayer.

2a. Tubulin tyrosylation and microtubule assembly: specificity of a detyrosylating enzyme for polymeric substrate (N. KUMAR and T. JONES): As reported here 2 years ago, tubulin tyrosylating enzyme has little or no activity toward assembled microtubules. Several years ago Caputto's laboratory reported that in brain extracts detyrosylation was favored by conditions promoting assembly, and more recently they showed that tyrosine was lost more rapidly from the previously assembled fraction (Arce et al. J. Neurochem. 31, 205). We had (last year's report) purified a brain detyrosylating enzyme (CPT) 500 fold, and although it is quite active on subunits and even urea-denatured tubulin, we now have definitive evidence that polymeric forms are preferred substrates.

If 4xp ^{14}C-tyrosylated tubulin was warmed for 30 minutes before adding CPT, the presence of stoichiometric (5 μM) podophyllotoxin during the preincubation inhibited the rate of detyrosylation by up to 90%, during the interval when the first 1/3 of tyrosine was being released. In the same experiment with PC-tubulin (assembly incompetent due to removal of MAPS with phosphocellulose) podophyllotoxin had no effect, but if MAPS fraction was added back the rate was increased 3 fold, and the increment was podophyllotoxin-sensitive.

Taxol, a complex low molecular weight plant product, has recently been shown to promote assembly in the absence of MAPS (and of GTP and Mg^{2+}). Taxol gave exactly the same result as MAPS.

Some other oligomeric structures also appear to be preferred substrates. Vinblastine inhibits assembly at 1 μM, induces paracrystals at 1 mM. and at about 10 μM has been reported to produce small (9s) oligomers. In the latter concentration range it consistently increased the rate of detyrosylation of

PC-tubulin. Maytansine, which binds to the same site as vinblastine but does not elicit oligomers, has not stimulated CPT at any concentration.

In relation to how CPT might act on such a large structure as a microtubule, i.e. on ends, like a zipper etc., we might mention the preliminary observation that detyrosylation takes place maximally early in assembly, and decreases at a rate > the rate of disassembly. The low specific activity of CPT in extracts (2% of ligase) suggests that optimal substrate or conditions may not yet have been identified.

More interesting is the possibility that in living cells tyrosylation and detyrosylation are coupled in some physiological way to cycling assembly and disassembly.

2b. Steady state transit times in treadmilling of T- and D-tubulin through microtubules (N. KUMAR and T. JONES): Many experiments have given variable results with both tubulin species, with average transit times, measured from the assembly end, ranging from a normal 12 hours down to 1½ hours. We are currently processing micrographs to determine whether this is related to few long vs many short microtubules. The indications are that with MAPS undamaged by the procedures involved in making T- and D-tubulin, both kinds of tubulin transit at the same 12 hour rate.

3. Paradoxical tyrosylation of membrane-bound tubulin (J. NATH): The paradox described last year arose from the observations that a) tubulin purified from a brain membrane fraction assayed as having no C-terminal tyrosine and b) tubulin isolated the same way after incubating brain mince with labeled tyrosine, or after intracerebral injection, appeared to have as much C-terminal radioactivity as did cytoplasmic tubulin. One resolution suggested last year was that the assay for (a) might be in error because of ATPase activity in the membrane fraction. The assay is based on the incremental amount of tyrosine fixed by tubulin-tyrosine ligase after pre-existing tyrosine has been removed by carboxypeptidase A (CPA). The ligase reaction is reversible and in the presence of ATP + ADP + P_i catalyzes both net tyrosylation and complete exchange of pre-existing tyrosine. If this happened there would apparently be no increment in the amount of tyrosine fixed, after CPA treatment. By incubating ligase with purified membrane-bound and cytoplasmic tubulins together we have shown this is not the case, because the assay still showed the same amount of pre-existing tyrosine as when cytoplasmic tubulin was tyrosylated separately.

A probable resolution of the paradox has now been suggested by the observation that the in vivo fixed radioactivity is not released by ligase in the presence of ADP + P_i, under conditions where 2/3 of the tyrosine fixed by crude extract or purified ligase is released. CPA released the radioactivity in a form that cochromatographed with tyrosine, so the result was not due to an in vivo fixation of a derivative which could be released by CPA but not by ligase. It is virtually certain that, if ligase can not remove tyrosine from this tubulin species, it also could not put it on, and this species would therefore assay as non-substrate tubulin (a portion, about ½ of both membrane and cytoplasmic tubulin, which can not be tyrosylated even after CPA treatment). The structural modification which makes this species unreactive with ligase would have to occur together with or after tyrosylation, or else the ligase specificity would have to be different in cells than it is in extracts.

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Tyrosine fixed in vivo in cytoplasmic tubulin was also not released by ligase in the presence of ADP + P_i. This represents a complication, since 20-40% of the substrate fraction of this tubulin always assays as having pre-existing tyrosine. Whether or not the paradox is resolved, this project should provide a way to get at the nature of non-substrate tubulin.

4a. The effect of chemotactic stimulation on tubulin tyrosylation in rabbit peritoneal leukocytes (J. NATH): Chemotaxis, the directed migration of cells in response to a chemical gradient, in this case of N-formyl-methionyl-leucyl-phenylalanine (fmlp), is associated with a reorganizaton of the cytoskeleton. We had previously found changes in tubulin tyrosylation to accompany comparable reorganizations that occur during neurite extrusion by differentiating neuronal cells, and during mitosis of HeLa cells. The change was an increase in the proportion of tubulin with pre-existing tyrosine, at the expense of the "non-substrate" species. Peritoneal leukocytes, although they have already migrated through the endothelium, are relatively quiescent: increasing states of activation are in the order: peripheral blood → peritoneal → chemotactic stimulated → phagocytic.

Ligase activity (0.01 unit/mg) was not demonstrably different in extracts of control and fmlp treated cells. Proportions of tyrosylated species of tubulin were the same, with somewhat more pre-existing tyrosine than we find in brain. This result is not certain, as vinblastine precipitation did not purify the tubulin effectively due to the low concentration (1% of soluble protein) of the latter.

Chemoattractant was found to increase the in vivo rate of labeled tyrosine fixation at the α chain C-terminus. After preincubation with protein synthesis inhibitors label was found only in α chain, and 70% could be released by CPA. The apparent rate increased 2-3 fold as soon as fmlp was added to the medium, and fixation did not plateau during the 120 minutes healthy lifetime of the cells. The increase was not due to increased transport of labeled tyrosine into the cells. The increase was abolished by every inhibitor of chemotaxis, specifically: carbobenzoxy-phenylalanyl-methionine, which blocks the fmlp receptor; nordihydroguaiaretic acid, which inhibits fmlp-induced Ca^{2+} influx; quinacrine, which inhibits phospholipase A_2; and deazaadenosine + homocysteine thiolactone, which inhibit phospholipid methylation. These results might suggest a link between tubulin tyrosylation and membrane phenomena, but of course each inhibitor shuts down the whole chemotactic process.

Methylation of specific protein glutamate γ-carboxyls and of phospholipid also occur immediately on exposure to fmlp, but within 5 minutes the methyl groups are removed again. The increased tyrosine fixation does not show this transient character. All known biochemical changes induced by fmlp are also induced by Con A, and con A treated leukocytes also showed increased tyrosine fixation. With cells preincubated for 30 minutes with concentrations of colchicine reported to block chemotaxis and the associated cytoskeletal rearrangement, fixation stimulation by fmlp was unaffected, but stimulation by con A was blocked.

4b. Tubulin tyrosylation in leukocytes from Chediak-Higashi syndrome patients (J. NATH): CHS is a rare autosomal recessive disease involving susceptibility to pyogenic infection and defects in polymorphonuclear leukocyte function. The rate of tyrosine fixation in tubulin α chain was determined in

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fmlp-stimulated, and unstimulated, peripheral blood leukocytes. Leukocytes from normal human controls required 10 times higher (10^{-8}M) fmlp than rabbit peritoneal leukocytes, to induce chemotaxis; the basal unstimulated rate of tyrosine fixation was slightly (1/3) lower. Relative rates of tyrosine fixation were as follows: control 1.0, control + fmlp 1.3, CHS 2.0, CHS + fmlp 4.0. In the last case (only) the amount of label fixed reached a plateau (at 90 minutes).

Ascorbate, which is empirically therapeutic, reduced fixation rates to control levels either when added to the leukocyte incubation medium, or administered orally to the CHS patients 2 hours before blood was drawn.

Ligase levels were the same in extracts of CHS and control leukocytes. Since turnover could also be limited by detyrosylation, we compared the rate of release of trichloroacetic acid soluble radioactivity from ^{14}C-tyrosylated tubulin. Radioactivity was released by control, but not by CHS, extracts. The release was not due to a detyrosylating enzyme, however, (see 2A), but to a particulate protease, since radioactivity from tubulin labeled with ^{3}H-leucine was released in parallel. Labeled actin was not digested.

5. Miscellaneous projects: During the last year's squid season at Woods Hole we made a final attempt to repeat the experiments reported from Sakai's laboratory on the effects of perfusing the axon with tyrosine and tubulin (last year's report). Axons were cleared with pronase, and then perfused with medium containing 500 mM KF until the action potential began to fail. No restoration of function was observed after adding to the perfusate: tyrosine + Mg^{2+} + ATP + cAMP, or the same + tubulin or purified ligase. We also perfused with maximally tyrosylated tubulin, without effect.

Proposed course of project:

Project 1. Experiments are in progress to determine whether MAPS arms periodicity, or the protofilament number, correlate with the different MAPS to tubulin stoichiometry in microtubules assembled from T- or D-tubulin. We would like to compare dynein arm binding also, since this shows more fine structure. We plan to prepare T and D nucleating fragments and compare the 2 types of tubulin for nucleation and elongation. In the liposome project we plan to identify the regions of tubulin that are embedded in the bilayer, and would like to extend this approach to biological membranes.

Project 2. We will ascertain whether there has been preferential loss of tyrosine from polymer when CPT acts on tubulin-microtubules at steady state. We hope to stabilize specific oligomeric structures (rings, sheets, ribbons, etc) to characterize CPT specificity. To study how CPT acts on polymer, we can see if it prefers ends by comparing rates with many short, versus a few long, microtubules.

Project 3. We need better cells than brain mince, and a better purification of the *in vivo* labeled tubulin, to eliminate background spontaneous detyrosylation. We would like to show directly that the tubulin that can be detyrosylated by ligase, also can not be tyrosylated by it. Isoelectric focussing may be helpful: it splits α chain into at least 5 bands not all of which are labeled with tyrosine *in vivo*. The general approach to the nature of non-substrate tubulin would be to find a cell-free system which could convert it to substrate tubulin, or vice versa. One could introduce *in vitro* labeled (i.e. substrate) tubulin into delicately permeabilized cells. Or one could work with homogenates closely approximating cells, seeking a system that could make ligase-refractory tyrosylated tubulin.

Project 4. The different effects of colchicine on tyrosine fixation in con A, as against chemoattractant, stimulated leukocytes may help in associating specific changes in cell morphology with accelerated tyrosine fixation. We would like to study the effects on tyrosine fixation of stabilizing microtubules with taxol, and of depolymerizing them with podophyllotoxin. We would like to characterize the stimulated fixation kinetically (is it turnover?), and in cell subcompartments. Half the leukocyte tubulin is particulate; this amount has been found elsewhere only in brain, where it has been simply attributed to there being so much membrane, in the form of myelin. Further along, this project may join with project 2 in asking whether assembly/disassembly and tyrosylation/detyrosylation are coupled.

Publications:

1. Nath, J., and Flavin, M.: Tubulin tyrosylation in vivo and changes accompanying differentiation of cultured neuroblastoma-glioma cells. J. Biol. Chem. 254: 11505-11510, 1979.

2. Nath, J., and Flavin, M.: An apparent paradox in the occurrence, and the in vivo turnover, of C-terminal tyrosine in membrane-bound tubulin of brain. J. Neurochem. 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00505-14 LCB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cytology of Acanthamoeba

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Blair Bowers	Research Biologist	LCB NHLBI
Other:	Claudia F. Bailey	Guest Worker	LCB NHLBI
	Enrico Cabib		LBM NIAMDD
	Rowena Roberts		LBM NIAMDD

COOPERATING UNITS (if any)

Laboratory of Biochemistry and Metabolism, NIAMDD

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Antibodies to two components of a membrane glycolipid in Acanthamoeba castellanii have been prepared. Indirect immunofluorescence methods were used to determine the distribution of the antigens in the plasma membrane and in internal membranes.

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Project Description

Objectives: Our major project is a study of how surface membrane taken in during endocytosis is handled by the cell and in what form it is returned to the surface. We are using as a model system a small amoeba, Acanthamoeba castellanii, a cell that has an unusually high rate of membrane recirculation associated with pinocytosis. The problem is most accessible through morphological techniques that examine the relevant membranes in situ, so that a variety of electron microscopic procedures are being utilized.

Methods employed: Standard immunological procedures were used to produce in rabbits, collect, and fractionate IgG antibodies made to membrane glycolipids. Antibody titers were determined by microagglutinin tests. Polyacrylamide gel electrophoresis and autoradiography of gels were used for study of fixed and embedded cells and for examination of isolated cell structures. Transmission and scanning electron microscopy were used for study of cell surfaces and surface labels. Fluorescence and phase-contrast microscopy were used for localization of specific antigens in intact cells, cell fractions and in sections of embedded material.

Major Findings:

1. We have obtained antibodies specific to 2 fractions of lipophosphonoglycan (LPG). LPG (a glycosphingolipid) is a major component of the Acanthamoeba plasma membrane. The two fractions are defined by their separation into two bands on polyacrylamide gel electrophoresis, and are designated by their relative mobilities as "fast" and "slow" LPG. The difference between these components has not been completely characterized, but at least the sugar content is not the same. The slow component contains glucose, mannose, and xylose and the fast component, glucose, mannose, and galactose (Dearborn et al., J. Biol. Chem. 251:2976, 1976). Antibodies made to these two fractions were not cross-reactive. Since the antigens are demonstrable in intact cells, it is likely that the antibodies were induced against the carbohydrate portions of the molecules. Radiolabeled antibodies to fast and slow fractions react with their respective fast or slow band and not with any other bands of solubilized whole cells run on polyacrylamide gels, suggesting that they are indeed specific for those fractions. We have examined the localization of the fast and slow LPG antigens in intact cells, in cell fractions, and in sections of embedded cells. Indirect immunofluorescent labeling of intact cells showed that antibodies to both components of LPG have the same, uniform distribution on the cell surface. Both antibodies also bind to the cytoplasmic side of isolated phagosomes, confirming our previous cytochemical evidence that carbohydrate portions of the molecules extend on both the extracellular side and the cytoplasmic side of the membranes. Isolated nuclei were not reactive, suggesting that the endoplasmic reticulum (which forms the nuclear envelope) also is not reactive. This finding is reasonable if in Acanthamoeba, as in many other cells, glycosylation of membrane components occurs in the Golgi apparatus. How the molecules are glycosylated on both sides of the membrane is an intriguing problem which may begin to be addressed if we are able to obtain high resolution localizations of these antigens.

In order to visualize LPG antigenic sites inside the cell, it was necessary to obtain sections of fixed and embedded cells in which the LPG antigenic sites were retained in a reactive condition. For this purpose we used gentle fixation with glutaraldehyde and a water soluble embedding medium, polyethylene glycol. The antigenic studies were demonstrated in sections with an indirect method that couples horseradish peroxidase (HRP) to the specific antibodies. The HRP can then be demonstrated cytochemically. In sections, the localizations of both LPG fractions to the plasma membrane and not to the nuclear envelope were confirmed. In addition, as might be expected, digestive vacuole membranes were labeled. An additional important observation was made. The contractile vacuole membrane, which is functionally and morphologically different from the plasma and vacuole membranes was strongly reactive to LPG antibodies. The very small vesicles found abundantly in the cytoplasm and related to the plasma membrane and vacuolar system, were not resolved with these light microscopic studies. The distribution of LPG in these small vesicles is important to determine since they are thought to be a major pathway of the membrane shuttle from vacuole to plasma membrane, but the development of electron microscopic methods for localization will be required.

We have also examined whether the specific antibodies might be suitable as markers to follow membrane transit in living cells. Cells were labeled with antibody at 0°, washed, reincubated, and the distribution of the antibody followed. Cells were fixed, permeabilized, and examined by indirect immunofluorescence at 10, 30, and 60 min after reincubation. By 10 min internal vacuoles showed a fluorescent rim. The vacuoles remained fluorescent for the duration of the experiment. During this time the label did not disappear from the surface. Virtually all internal vacuoles except the contractile vacuole became labeled, but fluorescence also appeared free in the vacuole in some cases. These observations directly demonstrate the exchangeability of vacuole and plasma membrane, but suggest that the specific antibodies are not an entirely stable label when applied to the living cell. In contrast to the behaviour of the antibodies to the LPGs, fluorescein-Con A(Fl-ConA) which presumably labels the same molecule as the antibodies, patches and caps on the cell surface. Some Fl-ConA is also endocytosed and appears to be stripped from the cell surface when internalized and is expelled from the cell as brightly fluorescent granules. The membrane of cells reacted with ConA, when examined in the electron microscope, shows considerable vesiculation. It appears that the membrane separates from the cell as small blebs, but tends to remain adherent to the surface, probably due to the cross-linking action of the ConA. This debris then accumulates as a cap. Thus ConA is much more damaging to the plasma membrane than are specific antibodies under the same conditions. We are attempting to examine the surface of cells treated with ConA and with antibodies at high resolution with replicas of frozen-etched cell surfaces in order to determine the reasons for this difference.

2. The cell wall of Saccharomyces cervisiae is formed by 3 polysaccharide polymers: mannan, glucan, and chitin. Mannan and glucan form the bulk of the wall material. Chitin is concentrated in the region of bud scars (remnant of the separation site between mother and daughter cell). It has been found previously that chitin occurs as a ring around the base of the developing bud and that

it forms the primary septum that separates the finished daughter cell from the mother cell. Last year we reported results that show chitin synthase itself is not localized, but is found generally distributed on the yeast cell membrane. Thus, there appeared to be a highly restricted spatial (and temporal) expression of chitin synthase activity. As part of a continuing collaboration with Enrico Cabib, aimed at understanding how the yeast cell controls chitin synthase, we have attempted this year to apply electron microscopic methods for chitin localization *in situ*.

Identification of chitin on thin sections allows more sensitive localization of the site and timing of chitin synthase activity than can be achieved with the combinations of selective extractions and chemical analyses used previously. We have used wheat germ agglutinin (WGA)-labeled colloidal gold as an EM probe. WGA binds specifically to n-acetylglucosamine, the chitin monomer. The results are preliminary, due to several technical problems, but the images already suggest a new finding - namely that cellular spatial control of chitin synthase has some slippage. The newly formed buds show no chitin deposition in the wall, but cells which have budded show variable amounts of label in the wall. At each budding cycle there appears to be some small amount of chitin synthesized randomly around the wall in addition to intense and morphologically well-defined deposition at the site of budding. This observation is relevant in defining working hypotheses for control mechanisms.

Proposed course of project: We will attempt to obtain localizations at the electron microscopic level of antibodies to *Acanthamoeba* membrane LPGs.

Publications:

Bowers, B. Morphological study of plasma and phagosome membranes during endocytosis in *Acanthamoeba*. J. Cell Biol. 84:246-260, 1980.

Molano, J., Bowers, B., and Cabib, E.: Distribution of chitin in the yeast cell wall. An ultrastructural and chemical study. J. Cell Biol., 85:199-212, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00506-05 LCB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Acanthamoeba myosins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Edward D. Korn	Chief	LCB NHLBI
Others:	Hana Gadasi	Visiting Fellow	LCB NHLBI
	Sueo Matsumura	Visiting Fellow	LCB NHLBI
	Jimmy Collins	Staff Fellow	LCB NHLBI
	Hiroshi Maruta	Visiting Associate	LCB NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Cell Biology

SECTION

Section on Cellular Biochemistry and Ultrastructure

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.25	PROFESSIONAL: 3.25	OTHER: 0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The three myosin isoenzymes, IA, IB and II, of Acanthamoeba castellanii also occur in related amoebae and seem to have different intracellular localizations: myosin IA and IB being preferentially localized at or near the periphery of the cell. Despite their unusual subunit composition, the isolated myosins seem to be the native forms of the molecules. The ATP-site and the phosphorylation-site on the heavy chain of myosin IA can be separated from each other by subtilisin digestion. Subtilisin digestion also produces lower molecular forms of myosin IA whose Mg-ATPase activity can be actin-activated without the phosphorylation that is required for the native enzyme. A highly actin-activatable form of myosin II has been isolated for the first time. It contains about 0.97 mol P/mol of heavy chain and its actin-activated ATPase activity is enhanced 3-4 fold by dephosphorylation to 0.35 mol P/mol of heavy chain. This is the first example of dephosphorylation, rather than phosphorylation, enhancing an actomyosin ATPase. Therefore, in the same cell the ATPase activities of actomyosin IA and IB are enhanced (derepressed) by phosphorylation of their heavy chains and the ATPase activity of actomyosin II is enhanced by dephosphorylation of its heavy chain.

PHS-6040
(Rev. 10-76)

Project Description

Objectives: Previously, we have shown that Acanthamoeba castellanii contains at least three myosin isoenzymes, each a product of a different structural gene. Myosin IA and IB have native molecular weights of about 150,000 and consist of one heavy chain (130,000 daltons for IA and 125,000 daltons for IB) and two light chains (17,000 and 14,000 daltons for IA and 27,000 and 14,000 daltons for IB). Myosin II has a native molecular weight of about 400,000 and consists of two heavy chains of 170,000 daltons and two pairs of light chains of 17,500 and 17,000 daltons. Peptide maps of the heavy chains of the three myosins show no similarities and the three enzymes are also distinguishable immunochemically. This year we had four major objectives.

(1) Cellular localization of the myosin isoenzymes. Indirect evidence had suggested that all three enzymes occurred in the same cells. Because this is the first example of myosin isoenzymes in a non-muscle cell and because of the unusually low molecular weight of their heavy chains it was important to establish that a single amoeba does contain all three isoenzymes, to show that the isolated proteins are not degradation products of the native molecules, to obtain some evidence for the localization of the several myosins within the cell, and to determine if similar enzymes occur in other cells. Progress towards each of these goals has been made this year.

(2) ATP site and phosphorylation site of myosin I. The Mg-ATPase activities of myosins IA and IB are actin-activated only when their heavy chains are phosphorylated. The isolated enzymes are non-phosphorylated but we have previously paritally purified a myosin I heavy chain kinase that specifically phosphorylated the 130,000-and 125,000-dalton heavy chains to give actin-activated enzymes. This year we initiated studies to determine the relationship between the phosphorylation site and the ATP-binding site, both of which we knew were on the heavy chains of the isoenzymes, and to determine whether phosphorylation is an activation or a derepression of the actin-activated ATPase activity.

(3) Actin-activatable myosin II. In contrast to myosin IA and IB, we have had great difficulty in obtaining a preparation of myosin II whose ATPase activity was actin activatable despite the fact that in other respects this isoenzyme more closely resembles myosins from other sources than do myosins IA and IB. A major goal this year was to isolate an actin-activatable myosin II and, if possible, to determine the basis of the regulation of actin-activation of myosin II.

(4) Acanthamoeba myosin III. The three isoenzymes just described account for about 90% of the total myosin-like ATPase activity in extracts of the amoeba. A fourth minor myosin-like ATPase fraction has consistently been observed in elution patterns from agarose gel columns. Another goal this year was to purify that activity, here referred to as myosin III, and to determine if it is related to myosin IA, IB or II or is yet another myosin isoenzyme.

Methods employed and findings:

(1) Cellular localization of the myosin isoenzymes. To obtain monospecific antibodies against the three myosin isoenzymes, the antibodies previously raised against the purified enzymes were used to immunoprecipitate each of the isoenzymes specifically from extracts of whole amoebae. The immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and, in each

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case, only one Coomassie blue-stainable band was found in the region of the heavy chains. The heavy chains were excised and used in new rabbits to raise antibodies directed specifically at the heavy chains of each of the three myosins isoenzymes. In this way, a contaminating polypeptide would have had to have been precipitated by the antibody directed against the purified mysoin and have had the same electrophoretic mobility as its heavy chain. To test the specificity of each of the antibodies, whole amoebae were boiled in SDS and the polypeptides separated by polyacrylamide gel electrophoresis. Individual lanes were transferred and covalently linked to diazobenzyloxymethyl paper, reacted with one of the three specific antibodies and then with ^{125}I-labeled protein A which reacts with the antibodies bound to the paper by reaction with the antigen. The location of any protein that reacts with the antibodies can then be detected by autoradiography. Antibodies to mysoin IA, IB and II heavy chain reacted exclusively with polypeptides of 130,000, 125,000 and 170,000 daltons, respectively, proving that each was specific and that the amoeba does not contain higher molecular polypeptides that are precursors of the isolated molecules.

These antibodies were then used for indirect immunofluorescence cytochemical studies. Amoebae were allowed to adhere to glass slide, made permeable with cold acetone, and exposed to one of the three antibodies followed by rhodamine-labeled goat anti-rabbit antibody. The cells were then photographed under phase contrast and fluorescence microscopy. Every cell in every field was found to react with each of the three antobodies proving that every cell contains all three myosin isoenzymes. Moreover, by this assay, myosins IA and IB seemed to be relatively more concentrated at, or near, the cell periphery while myosin II was largely excluded from this area.

The differential localization of the three myosin isoenzymes was confirmed in several ways. First, sections of glutaraldehyde-fixed cells were reacted with one of the three antibodies, then with goat-antirabbit antibody coupled to horseradish peroxidase, and the location of the antibodies determined by the peroxidase reaction. The results were the same as for the immunofluorescence studies. Then, plasma membranes were isolated by techniques developed earlier in this laboratory. The isolated plasma membranes were positive for myosins IA and IB and negative for myosin II in the indirect immunofluorescence assay and were greatly enriched in the heavy cahins of myosins IA and IB when analyzed by SDS-polyacrylamide gel electrophoresis and ^{125}I-protein A staining.

Indirect immunofluorescence assays demonstrated that the presence of proteins similar to the three myosin isoenzymes in three other amoebae: another strain of Acanthamoeba castellanii, Acanthamoeba astronyxis and Nigleria gruberii.

(2) ATP site and phosphorylation site of myosin I. Exposure of myosin IA and [^{3}H]ATP to ultraviolet light at 4° for approximately 1 hour results in the incorporation of about 0.1 mol of nucleotide/mol of myosin. Evidence that this covalent labeling involves the active site of the enzyme includes (a) the enzyme is labeled with [α-^{32}P]ATP but not with [γ-^{32}P]ATP suggesting that the bound ATP is hydrolyzed to ADP, (b) labeling also occurs with other substrates

or products such as GTP, UTP and ADP but not with AMP, (c) labeling is inhibited by pyrophosphate, which inhibits enzymatic activity, but not by phosphate which does not inhibit the enzyme, (d) the incorporated label is in the form of ADP so the ATP has been hydrolyzed. Labeling with UTP is more efficient, about 0.5 mol/mol. Similar labeling occurs with mysoin II and smooth muscle myosin. SDS-polyacrylamide gel electrophoresis and autoradiography reveals that all of the label is incorporated into the heavy chains of all of the myosins. The production of labeled peptides when ATP-labeled myosin IA was degraded with subtilisin was followed by SDS-polyacrylamide gel electrophoresis. Radioactivity sequentially appeared in peptides of 130,000 (the heavy chain), 115,000, 28,000 and 16,000.

When myosin IA was incubated with myosin I heavy chain kinase and [γ-^{32}P]ATP only the heavy chain became labeled. When degraded by subtilisin under conditions identical to those used for the ATP-labeled enzyme, radioactivity appeared sequentially in peptides of 130,000 (heavy chain) 115,000, 87,000 and low molecular peptides at the front. Thus, a 115,000 dalton peptide retains both the ATP-site and the phosphorylation site but the subsequent 87,000-dalton fragment that contains the phosphorylation site does not contain the ATP-site and the 28,000-dalton peptide that contains the ATP-site does not contain the phosphorylation-site. It may be that the 115,000-dalton peptide is cleaved to the 87,000 and 28,000 dalton peptides whose combined molecular weight is 115,000. Separation of the ATP-labeled site from the phosphorylated-site can also be accomplished by CNBr cleavage (at methionine residues) followed by chromatography on Sephadex G-25.

Limited digestion of myosin IA by subtilisin to fragments of "native molecular weights in the range of 17,000-68,000 in which the largest major peptide is 28,000 daltons can be accomplished with no loss of any of its enzymatic activities including actin-activated Mg-ATPase. When non-phosphorylated myosin is treated in this way with subtilisin, the digestion products are fully activatable by actin even without phosphorylation although the original myosin was not actin-activatable. This strongly suggests that enhancement of actin-activation by phosphorylation of intact myosin I is a derepression which can also be accomplished by proteolytic removal of the non-phosphorylated peptide.

(3) Actin-activatable myosin II. An excellent procedure has been developed which routinely produces highly actin-activatable myosin II in about 50% yield, about 10 mg/100 g wet weight amoebae. The procedures involve dialysis of the crude extract at low ionic strength to precipitate actomyosin II, dialysis against an appropriate buffer to depolymerize excess actin, solubilization of the myosin in KCl containing ATP, chromatography on agarose columns, and removal of traces of nucleic acid by DEAE-cellulose. The product has the same high Ca-ATPase and low K, EDTA-ATPase activities as the enzyme prepared by other procedures had but, for the first time, its Mg-ATPase activity is highly actin-activated to an activity of about 0.2 μmol/min/mg., a value as high as has been obtained for any non-muscle myosin other than Acanthamoeba myosins IA and IB. Myosin II prepared by this procedure is indistinguishable from the product of the other procedures in native molecular weight on agarose columns or in its subunits as judges by SDS-polyacrylamide gel electrophoresis and isoelectric focussing of its light chains.

From past experience, there were two obvious possibilities to explain the actin-activation of these preparations of myosin II; (a) heavy chain phosphorylation as for Acanthamoeba myosins IA and IB, or (b) light chain phosphorylation as for smooth muscle and vertebrate non-muscle myosins. Heavy chain phosphorylation seemed unlikely because we knew that previous non-actin-activatable preparations of myosin II were phosphorylated on their heavy chains. Therefore, we isolated myosin II by the new procedure from amoebae grown in the presence of ^{32}P and analyzed the distribution of radioactivity by autoradiography of SDS-polyacrylamide electrophoretic gels. Although the preparation was highly actin-activatable and the heavy chains were radioactive, the light chains contained no radioactivity. Therefore, the actin-activation of myosin II Mg-ATPase did not seem to involve phosphorylation of its light chains.

On the assumption that it was, as for myosins IA and IB, the heavy chain phosphorylation that allowed actin-activation of myosin II, and that previous preparations were inactive for reasons unrelated to their state of phosphorylation, we incubated myosin II with commercial potato acid phosphatase and with a smooth muscle myosin phosphatase provided by Drs. Pato and Adelstein. Dephosphorylation was monitored by the loss of radioactivity as measured by quantitative scans of autoradiograms. Both enzymes could remove almost all of the radioactivity form ^{32}P-myosin II. But instead of a loss in actin-activation there was an enhancement of actin-activation in parallel with the loss of radioactivity. The dephosphorylated myosin was actin-activated to specific ATPase activities about 3-fold greater than the original myosin to final activities about 0.75 μmol/min/mg. Careful analysis of Coomassie blue-stained electrophoretic gels showed no evidence of proteolysis and inhibitors of the phosphatase activity showed identical inhibition of the enhancement of the actin-activated myosin ATPase.

Direct chemical analysis of the phosphate content of actin-activatable myosin II gives a value of about 1.94 mol/mol of myosin or 0.97 mol/mol of myosin heavy chain. Dephosphorylation lowers the value to about 0.35 mol/mol of heavy chain. Myosin II prepared by methods that lead to an essentially non-actin-activatable enzyme contains a higher content of phosphate, about 3.8 mol/mol of myosin or 1.4 mol P/mol of heavy chain. It, too, becomes highly actin-activatable when dephosphorylated to 0.31 mol P/mol of heavy chain. From these data, it seems possible that fully phosphorylated myosin might contain 2 mol P/mol heavy chain and not be activated by actin. Myosin II prepared by a procedure that leads to its partial dephosphorylation would be partially actin-activated and actin-activation would be enhanced by removing additional phosphates with phosphatases.

This is the first example of a myosin in which phosphorylation decreases, rather than increases, actin-activation of its Mg-ATPase. It is particularly intriguing because phosphorylation of the other myosin isoenzymes in the same amoeba has exactly the opposite effect.

(4) Acanthamoeba myosin III. The protein responsible for the remaining 10% of myosin-like ATPase activity in Acanthamoeba has been highly purified in

small yield. It is clearly a single-headed enzyme, like myosins IA and IB, with a native molecular weight of about 210,000, but has a heavy chain of about 165,000 daltons, similar to but different from, the 170,000-dalton heavy chain of myosin II. The enzyme is not sufficiently pure to be sure of its light chain composition. The most striking feature of this enzyme is its very high ATPase activities. The Ca-ATPase and K,EDTA-ATPase activities are 7 and 1.5 μmol/min/mg, respectively, which are in the same ratio as for myosin II but about 10 times higher. The actin-activated Mg-ATPase activity of myosin III is about 28 μmol/min/mg which is about 10 times higher than has been observed for any preparation of myosin II and 3 times higher than for myosins IA and IB. We could find no evidence for the formation of myosin III by degradation of radioactively labeled (^{35}S-methionine) myosin II but very high concentrations of antibodies raised against myosin II heavy chain do inactivate the ATPase activity of myosin III. At this time, we do not know if myosin III is a fourth Acanthamoeba myosin isoenzyme or a degradation product of myosin II.

Proposed course of research

Although the relative amount of time to be spent on each has not yet been determined, we hope to pursue most of the same problems next year. More evidence, perhaps by cytochemistry at the electron microscopic level, will be gathered for the differential localization of the myosin isoenzymes. Efforts will be made to study the physical properties of myosins IA and IB, in particular whether they can form bipolar filaments, the nature of their interaction with F-actin, and whether they can bind to cell membranes. Studies will be undertaken to determine the number and site of all possible phosphate groups on the heavy chain of myosin II. We will try to isolate the kinase and phosphatase from the amoeba that are responsible for regulating the state of phosphorylation of this enzyme. We will examine the nature of the Ca-sensitivity of myosin II for which we have some evidence.

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ANNUAL REPORT OF THE
LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1979 to September 30, 1980

As in the recent past, research in the Laboratory of Cellular Metabolism is largely concentrated on the enzymes responsible for the synthesis and degradation of cAMP and cGMP through which many hormones, drugs, and other agents influence cellular functions. In the Section on Cellular Pharmacology, studies of histamine metabolism and action along with investigations of the inflammatory process and anti-inflammatory drugs are continuing.

1. Cyclic Nucleotide Phosphodiesterases

We reported last year that, in addition to the three major types of soluble phosphodiesterases found in many tissues, there is a fourth form in rat liver. This probably had not been detected earlier, because with the procedures widely used for initial fractionation it is not separated from the calmodulin-dependent phosphodiesterase. We have now completed characterization of this enzyme. In molecular size, kinetic properties, failure to be activated by calmodulin, and effects of certain proteases on its activity, it differs from the other soluble liver phosphodiesterases. We have shown that this phosphodiesterase is present in cultured hepatoma (HTC) cells, where its activity is decreased by glucocorticoid treatment. No calmodulin-sensitive phosphodiesterase was detected in the HTC cells, although they apparently contain calmodulin.

The calmodulin-sensitive phosphodiesterase has now been purified 3600- to 4200-fold from bovine brain supernatant by two different methods. Both types of preparations show a single major peptide (58,000 daltons). Enzymes purified by both methods have the same specific activity in the presence of calmodulin, but one is activated six- to tenfold by calmodulin and the other only three- to fivefold. The latter enzyme appears to contain closely related isozymes, two major ones with isoelectric points of 5.45-5.50 and 5.60-5.65. Limited proteolysis with α-chymotrypsin produced a catalytically active peptide of ∿ 40,000 daltons. It did not bind to calmodulin-Sepharose nor was it activated by calmodulin, suggesting that the calmodulin-sensitive domain has been removed. We are now attempting to prepare monoclonal antibodies to the phosphodiesterase with the hope of obtaining antibodies specific for different domains in the protein which should be useful for their isolation and characterization as well as for subcellular localization of the enzyme. An antibody specific for the calmodulin-binding portion of the phosphodiesterase might also interact with other calmodulin-binding proteins. The method of target theory analysis was used in collaborative studies to estimate the molecular size of the phosphodiesterase. Samples were subjected to high energy ionizing radiation for varying periods and the loss of enzymatic activity determined. The functional size of the catalytic moiety estimated in this way was 60,000 daltons, irrespective of whether purified enzyme, gel filtration fractions, or crude supernatant was irradiated. The same value was observed when gel filtration fractions of M_r range 60,000 to 70,000 or 120,000 to 150,000 daltons were used.

These data are consistent with the view that the active enzyme unit is a monomer, although associated forms may be present in solution.

In collaboration with scientists in NIMH, we investigated the effects of carboxymethylation on the function of calmodulin. Calmodulin proved to be, relative to other purified proteins that have been tested, an excellent substrate for carboxymethylase. Its ability to activate phosphodiesterase was decreased by carboxylation. As both the carboxymethylase and demethylating enzymes are present in many cells, this type of covalent modification could be involved in regulation of the activity of phosphodiesterase (and perhaps other enzymes that are activated by calmodulin).

2. Adenylate Cyclase

Work in this area has been focussed on the mechanism by which choleragen activates adenylate cyclase and the GTP-binding protein that appears to be ADP-ribosylated by the toxin. We have also, in collaboration with scientists at Johns Hopkins University and NINCDS, further studied the role of ganglioside G_{M1} in toxin binding to the cell surface. 3T3-L1 cells (mouse embryo fibroblasts cloned by Green and Kehinde) differentiate in culture into cells with morphology and other characteristics typical of adipocytes. It was shown that G_{M1} is present in 3T3 fibroblasts but detectable only with special methodology in mouse adipocytes. During differentiation of 3T3-L1 cells, we found a 50 to 60% decrease in total ganglioside content and a 75 to 85% decrease in G_{M1}. In both L1 fibroblasts and differentiated adipocytes, G_{M1} represented $< 5\%$ of the total ganglioside. There was an inverse correlation between the percentage of adipocytes in a cell population and total ganglioside or G_{M1} content as well as surface G_{M1} estimated by choleragen binding. It appears that changes in G_{M1} content (choleragen binding) may be a useful marker for differentiation in these cells.

We have reported that choleragen and its A subunit exhibit NAD glycohydrolase as well as ADP-ribosyltransferase activity. Others have recently claimed that the former activity is due to a contaminating enzyme. The ability of choleragen to catalyze NAD hydrolysis is relevant to its mechanism of action; it demonstrates that the toxin can activate the ribosylnicotinamide bond in the absence of an acceptor more complex than water. We, therefore, confirmed our earlier observation that the purified A_1 peptide of choleragen (liberated from the A subunit by reduction of the single disulfide bond that links it to the A_2 peptide) possessed NAD glycohydrolase activity not detected in the A_2 peptide or the B subunit. All of the activity in our choleragen preparation cochromatographed with the A_1 peptide, the ADP-ribosyltransferase activity, and the ability to activate adenylate cyclase. Alkylated A_1 peptide was as active in both the NAD-glycohydrolase and ADP-ribosyltransferase assays as unmodified A_1. Based on a common sequence involving the cysteine and two adjacent amino acids in the A_1 peptide and the α-subunit of glycopeptide hormones, Ledley et al. suggested that toxin and the hormone might have similar mechanisms of action. As an unmodified cysteine is not required for activity of the A_1 peptide, however, it is unlikely that the sequence homology with the α-subunit occurs in a critical region or is related to an ability to catalyze similar reactions.

We have now purified to homogeneity an ADP-ribosyltransferase from turkey erythrocytes which appears to be a glycoprotein of molecular weight 28,000. With arginine methyl ester as the ADP-ribose acceptor, specific activity of the transferase is considerably higher than that of choleragen. Although both catalyze the same model reactions, they differ in their relative abilities to use different purified proteins or proteins in tissue fractions as ADP-ribose acceptors and in their capacities to use NADP in place of NAD. As the transferase is inhibited by thymidine, nicotinamide, and theophylline, some effects of these compounds on cells may be due to inhibition of ADP-ribosylation. We found earlier that the transferase (plus NAD) activated brain adenylate cyclase but have now shown, in collaborative studies, that its effects in other cyclase systems differ from those of choleragen. Thus, the role of the endogenous ADP-ribosyltransferase in cellular metabolism remains to be defined.

Others have found that several proteins in cell membranes can be ADP-ribosylated by choleragen; the principal one has a molecular weight similar to that of the GTP-binding protein component of the adenylate cyclase. We showed last year that the presence of GTP is required for activation of adenylate cyclase (ADP-ribosylation), for stabilization of the activated enzyme, and for expression of maximal catalytic activity in the assay. We have now investigated the effects of GTP on choleragen-catalyzed ADP-ribosylation. With [^{32}P]NAD as substrate, GTP increased ADP-ribosylation of numerous proteins in a fraction from bovine thymus. Other nucleoside triphosphates were less effective and GDP, GMP, or cGMP were ineffective. ADP-ribosylation of several purified proteins was, depending on the protein, either increased, decreased, or unaffected by GTP. Thus, enhancement by GTP of its choleragen-catalyzed ADP-ribosylation is itself insufficient to implicate that protein in adenylate cyclase regulation. The fact that choleragen can ADP-ribosylate a wide variety of proteins is consistent with the possibility that intoxication results in the covalent modification of more than one cellular protein and perhaps alters the activity of enzymes in addition to adenylate cyclase. To begin to define the role of the GTPbinding protein in the adenylate cyclase system, we are purifying it from porcine erythrocyte membranes, which have virtually none of the catalytic component of the cyclase. The GTP-binding protein is assayed by reconstitution with membranes from a lymphoma cell variant that lacks this protein; in its absence, the catalytic component, which the cells do not lack, is inactive. The GTP-binding protein, partially purified from detergent extracts with ion-exchange, hydrophobic, and molecular sieve chromatography, has an apparent molecular weight (gel filtration) of 130,000.

3. Guanylate Cyclase

Following an early step in purification, the guanylate cyclase from rat liver requires for maximal activity the addition of a component(s) separated at this stage. Numerous earlier attempts to purify this "activator" using standard methods for protein fractionation were unsuccessful. We have now found that "activator" survives exposure to 0.5 N NaOH for hours ~ 20° and after this treatment can be extensively purified using ion-exchange chromatography and gel filtration. Products of the reactions catalyzed by highly purified preparations of soluble guanylate cyclase from rat liver were

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identified and quantified with ^{31}P NMR spectroscopy. Utilization of this technique necessitated modification of the standard assay conditions. Higher concentrations of enzyme and substrate, Mg^{2+} instead of Mn^{2+}, and longer incubation times (up to 46 h) were employed. Revision of our reported procedure for purification of the cyclase provided an enzyme with specific activity higher than those of our earlier preparations. ^{31}P NMR spectra obtained during incubation of this enzyme showed that the rates of GTP disappearance and cGMP accumulation were constant for ~ 16 h but indicated the presence of inorganic pyrophosphatase. This was removed by preparative electrophoresis yielding enzyme with specific activities (900-1300 nmol/min/mg protein) higher than those reported for mammalian guanylate cyclases. With this preparation, cGMP and PP_i in amounts equivalent to the amount of GTP consumed were the only products detected, consistent with the presumption that the guanylate and adenylate cyclase reactions are analogous. Using the highly purified enzyme, we are now examining in detail the effects of thiols and oxidation or blockage of sulfhydryl groups on catalytic activity.

4. Regulation of Lipid Metabolism in Mammalian Cells

Studies of fatty acid metabolism in cultured human fibroblasts and skeletal muscle cells have been initiated this year. A number of myopathies and neuropathies are associated with the abnormal deposition of lipid, and in certain genetically transmitted diseases there is a striking accumulation of long-chain fatty acids normally present in trace amounts, e.g., phytanic acid in Refsum's syndrome, the biochemical basis of which was elucidated several years ago, largely through work in this laboratory. Increased amounts of hexacosanoic acid are found in tissues of patients with adrenoleukodystrophy or adrenomyeloneuropathy. We are synthesizing the ^{14}C-labeled fatty acid to investigate its metabolism by fibroblasts from patients with these diseases in collaboration with scientists from NICHHD. Carnitine is a cofactor in mitochondrial fatty acid oxidation. Several patients with a myopathy characterized histologically by the presence of intracellular lipid droplets in skeletal muscle have been described. The carnitine content of the muscle is low and the name of "carnitine deficiency" has been applied to this syndrome, although it is not clear that the patients are in fact carnitine "deficient" in the usual sense or that the disease has a single etiology. We have found no abnormality in carnitine uptake or effects on palmitic acid oxidation in fibroblasts from two patients who are being studied by investigators in NINCDS. Experiments with skeletal muscle cells are in progress. We have found that dexamethasone stimulated palmitate oxidation in fibroblasts and this effect was observed also when oxidation was increased by carnitine. The reported beneficial effect of glucocorticoid therapy in some patients with "carnitine deficiency" may be related to its ability to enhance fatty acid oxidation.

It is generally recognized that the rate of cholesterol synthesis in many mammalian cells is controlled by the activity of hydroxymethylglutaryl Coenzyme A (HMGCoA) reductase, which is subject to negative feedback regulation by cholesterol. We have investigated the possibility that other enzymes in the pathway of cholesterol synthesis might be subject to similar control and reported last year that mevalonate kinase activity in fibroblasts was altered in parallel with HMGCoA reductase in response to serum lipoproteins, although the changes were of lesser magnitude. We have recently found similar effects on mevalonate pyrophosphate decarboxylase activity. Like HMGCoA reductase, mevalonate kinase activity was increased by incubation of cells with insulin but decarboxylase activity was unchanged. Serum low density lipoprotein (LDL) reduces HMGCoA reductase (and cholesterol synthesis) as a result of its interaction with specific receptors on the cell surface. Antibodies to the LDL receptor could be useful for quantification, localization, characterization, and/or purification. Monoclonal antibodies would be particularly advantageous for these purposes. We have prepared hybridomas by fusion of spleen cells from an immmunized mouse with myeloma cells and are now screening to select and clone those hybrids that are producing antibodies to the LDL receptor.

5. Mechanism of Action of Anti-Inflammatory Drugs

We showed earlier that nonsteroidal anti-inflammatory drugs inhibit proliferation of cultured hepatoma (HTC) cells and fibroblasts. Cells are inhibited in the G_1 phase of the cell cycle and, on removal of indomethacin, resume growth in synchrony. Alterations in amino acid transport accompany these changes. The rate of transport of methylaminoisobutyric acid (MeAIB), a substrate for the "A" transport system, reaches a minimum 3 to 4 h after addition of indomethacin and returns to normal within 4 h following its removal. Kinetic studies this year indicated that the anti-inflammatory drugs did not alter affinities for amino acid or Na^+ but rather appeared to decrease the number of functional carriers in the cell membrane. Uptake of MeAIB occurred through low and high affinity components. The latter was prominent at all stages of growth of transformed cells but in nontransformed cells tended to disappear as cultures approached confluency. It was selectively inhibited by indomethacin. In both transformed and nontransformed cells, the drug shifted the pattern of amino acid transport to that observed in confluent nontransformed cells, i.e., uptake was largely through the "L" and low affinity "A" systems. These changes were correlated with effects on cell growth. Thus, the anti-inflammatory drugs may be useful in study of the cell cycle and associated changes in amino acid transport. Similar effects of indomethacin were observed in rat lymphocytes. A high affinity component of the "A" system was selectively inhibited when indomethacin was added to the cells or when it was administered to rats before harvesting lymphocytes, consistent with the possibility that the drug may influence amino acid uptake by cells *in vivo* as well as *in vitro*.

As a counterpart to studies with cultured cell, we are using as models of inflammation carrageenan- and dextran-induced pleurisy in rats. Carrageenan induces an intense, short-lived inflammation, characterized by accumulation of large numbers of neutrophils. Last year we found that indomethacin did not interfere with the production or release of white cells by bone marrow and lymphoid organs but prevented recruitment from the blood stream. We have now shown that appearance of serum proteins in the pleural cavity and generation of chemotactic activity occurs before neutrophil infiltration. Indomethacin aborts these reactions whether given before or after the onset of cell accumulation. In contrast to carrageenan, which does not cause degranulation of mast cells or accumulation of large amounts of fluid, higher molecular weight dextrans induced extensive degranulation of mast cells and edema shortly after injection into the pleural cavity; relatively few neutrophils were accumulated. With low molecular weight dextrans, the converse occurred; there was little degranulation of mast cells (or early edema) but extensive infiltration of neutrophils. Dextrans of intermediate size produced both responses. Indomethacin blocked infiltration of neutrophils but had no effect on the edema. It is concluded that histamine release and neutrophil infiltration are independent phenomena (although both may occur simultaneously) and that indomethacin has no effect on permeability changes that result from mast cell degranulation.

6. Role of Histamine in Gastric Secretion and in Brain

Histamine is a physiological mediator of gastric secretion, but little is known about its storage, mechanism of release, or inactivation in the gastric mucosa. The location of gastric histamine differs in different species. We have studied highly purified cell fractions from gastric mucosa prepared by Dr. Andrew Soll in collaboration with Morton Grossman at the Wadsworth V. A. Hospital in Los Angeles. In rat, rabbit, and dog, histamine and histidine decarboxylase are localized in a single type of cell. In the dog, this cell has a morphology characteristic of a mast cell and lies in the lamina propria in close proximity to the parietal cell. As a major portion of the mucosal histamine methyltransferase activity is associated with the parietal cells, this could explain the failure to detect free histamine in the gastric juice and the circulation in the dog. In the rat, the histamine-containing cell, situated in the gastric gland itself but at some distance from parietal cells, has the characteristics of an enterochromaffin cell. It contains abundant DOPA and histidine decarboxylase activities and has many of the properties of an "APUD" cell. Rat gastric mucosa, unlike that of the dog, contains no histamine methyltransferase activity, which may account for the appearance of histamine in rat urine during cholinergic- or gastrin-stimulated gastric secretion. Although these studies have provided useful information on differences in histamine storage and metabolism in different species, in extensive studies, freshly isolated histamine-containing cells from rat and dog have proved refractory to a wide range of histamine-releasing agents. We recently found, however, that after 24 h in culture mast cells from dog liver and mucosa released histamine on exposure to Ca^{++} ionophore or histamine-releasing peptides. Thus, it is now possible to investigate the mechanism of histamine release from these cells.

There is much circumstantial evidence that histamine is a neurotransmitter in mammalian brain. Although histamine is localized in regions that represent phylogenetically older parts of the brain, there has been no systematic study of its occurrence in primitive vertebrates. Our studies with 12 representative species indicate that histamine has been present in brain throughout vertebrate evolution. In fish and amphibia that do not have a true cerebral cortex (neopallium) or well-developed cerebellum, histamine levels were especially high in neural tissue and low in most peripheral tissues. In dogfish, histamine was present throughout the brain stem as well as in the spinal cord but was concentrated in the midbrain (hypothalamus) region; a large fraction of this was recovered in washed synaptosomal preparations. Histamine methyltransferase activity was high in the central nervous systems

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of all species; diamine oxidase activity was found also in some fish and amphibia. These findings are consistent with the view that histamine is a neurotransmitter and that, at least in primitive vertebrates, histaminergic neurones innervate regions of the forebrain associated with sensory stimuli (olfactory and optic lobes) which constitute a major portion of the brain mass in fish and amphibia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00603-06 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Cyclic Nucleotide Phosphodiesterase Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
Drs. Claude Gagnon and Fusaro Hirata, Lab. of Biochemical Pharmacology, NIMH; Drs. Brent Reed and M. Daniel Lane, Dept. of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore

LAB/BRANCH
Cellular Metabolism

SECTION
Biochemical Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work with the calcium-dependent cGMP phosphodiesterase is currently focused on mechanisms for regulation of calmodulin activity. Cyclic nucleotide phosphodiesterases in several tissues have been studied to characterize their regulatory properties. In other studies, we have found that with the differentiation of 3T3-L1 fibroblasts into cells having morphological and biochemical characteristics of adipocytes is the appearance of a particulate phosphodiesterase which exhibits a high affinity for cAMP and cGMP.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To characterize the multiple forms of cyclic nucleotide phosphodiesterases that catalyze the degradation of cAMP and cGMP in mammalian tissues and their relationships to each other. To elucidate the mechanisms through which the activities of these enzymes are regulated, e.g., by hormones, ions, or proteolysis and expressed in various cell types.

Methods Employed: Cyclic nucleotide phosphodiesterase activity is assayed by published methods modified in this laboratory. For purification of phosphodiesterases and regulatory proteins (e.g., calmodulin), ion exchange chromatography, gel filtration, preparative electrophoresis and affinity chromatography are used as needed.

Major Findings: Regulation of Calmodulin Activity. An enzyme, carboxyl-methylase, can covalently modify proteins by catalyzing the methylation of free carboxyl groups. Calmodulin, a highly acidic protein (pI $\sim$ 4.0) with high glutamic acid content, is a good substrate for the protein carboxyl-methylase. On a molar basis, methylation of calmodulin was almost twice that of luteinizing hormone, one of the most effective substrates previously known for protein carboxyl-methylase. Methylation of calmodulin reduced its capacity to activate phosphodiesterase.

Regulation of Phosphodiesterase Activity in 3T3-L1 Fibroblasts. 3T3-L1 fibroblasts under the influence of isomethylbutylxanthine and dexamethasone in the presence or absence of insulin accumulate triglyceride and take on the appearance of fat-laden adipocytes. During the course of this differentiation, there is a marked increase in the activity of a phosphodiesterase with high affinities for cAMP and cGMP. This enzyme was partially purified after solubilization of the particulate fraction in Triton X-100 by chromatography on DEAE-Biogel in a gradient of NaCl, and no evidence for separation of the two activities was obtained.

Significance to Biomedical Research: 1) Knowledge of properties of cyclic nucleotide phosphodiesterases and their regulation is necessary to understand mechanisms for control of cAMP and cGMP content in cells and tissues such as cardiac muscle and lung.

2) Knowledge of regulation of calmodulin and calmodulin-sensitive phosphodiesterase should facilitate understanding of Ca^{++} regulation of other enzyme systems, e.g., adenylate cyclase.

Proposed Course: 1) Complete characterization of various forms of cyclic nucleotide phosphodiesterases, e.g., the interrelationships between and localization of these forms.

2) Elucidate mechanisms for regulation of activity and for differential expression of specific forms of cyclic nucleotide phosphodiesterases in different types of cells.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00606-09 CM

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of cAMP Content of Cultured Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
OTHERS:	Su-Chen Tsai	Research Chemist	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

Fusaro Hirata, Donna Barese, and Julius Axelrod, Laboratory of Clinical Science, NIMH

LAB/BRANCH

Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.6	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cAMP content of cultured human fibroblasts is increased by incubation of cells with several effectors, e.g., bradykinin, PGE_1, isoproterenol, and choleragen. Bradykinin presumably produces its effects by stimulation of prostaglandin synthesis; isoproterenol and PGE_1 by interacting with specific receptors "coupled" to adenylate cyclase; choleragen by promoting the ADP-ribosylation of a regulatory component of the cyclase system.

Cellular responsiveness to these effectors can be independently altered by incubation of cells with agents, such as glutamine, indomethacin, dexamethasone, and choleragen

In preliminary experiments, bradykinin was found to increase cGMP but not cAMP content of cultured pig aortic smooth muscle cells.

Project Description:

Objectives: To elucidate the mechanisms through which several hormones, other physiological "messengers" and drugs influence the synthesis and degradation of cyclic nucleotides in cultured cells. Cultured cells serve as excellent model systems of relatively homogeneous populations of cells which can be grown and studied under defined conditions.

Methods Employed: Cultured cells grown under standard conditions; cAMP and cGMP purified by techniques developed in the laboratory and assayed by commercially available radioimmunoassay.

Major Findings: Regulation of cAMP content. Cultured fibroblasts respond to bradykinin, isoproterenol, prostaglandin E_1, and choleragen with an increase in cAMP.

This effect of bradykinin is enhanced by fetal calf serum, glutamine, and increases dramatically with duration of subculture. Duration of subculture has little effect on the response of PGE^1 but markedly reduces the response to isoproterenol.

Anti-inflammatory agents, e.g., dexamethasone and indomethacin, reduce responsiveness to bradykinin, presumably by inhibiting prostaglandin production. Dexamethasone increases responsiveness to isoproterenol and PGE_1.

Prolonged incubation with bradykinin reduces responsiveness to this agonist. Prolonged incubation with PGE_1 reduces responsiveness to bradykinin and PGE_1 and, to a lesser extent, isoproterenol. Brief incubation with choleragen increases responsiveness to low concentrations of PGE_1, bradykinin, and isoproterenol. Longer incubations decrease responsiveness to isoproterenol but not to bradykinin.

Regulation of cAMP Content. Cultured smooth muscle cells respond to bradykinin and histamine with a rapid (20 sec) increase in cGMP but not cAMP. PGE_1 increases cAMP but not cGMP in these cells.

Mechanism of Action of Bradykinin. In human fibroblasts, bradykinin increases phospholipid methylation, alters Ca^{++} flux, and promotes release of arachidonic acid from phospholipid, prostaglandin synthesis, and synthesis of cAMP. We have attempted to identify steps required for cAMP generation in response to bradykinin by use of selective inhibitors. Inhibition of prostaglandin synthesis by indomethacin or inhibition of phospholipase A_2 and arachidonic acid release by quinacrine inhibits cAMP production. Putative inhibitors of phospholipid methylation neither completely inhibit phospholipid methylation nor bradykinin-stimualted cAMP production.

Significance to Biomedical Research: Several hormones, neurotransmitters and other humoral agents exert effects on cells by altering metabolism of cAMP and/or cGMP. Various effectors activate adenylate cyclase by different mechanisms, and fibroblasts are a good model system in which to determine how responsiveness to hormones and other humoral agents can be regulated both independently and in a coordinate manner. Fibroblast may be particularly useful for learning how cell function is influenced by a locally generated effector, i.e., bradykinin.

Proposed Course: Continued study of regulation of cAMP and cGMP content by bradykinin and other effectors.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00607-07 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cyclic GMP Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Su-Chen Tsai	Research Chemist	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	1.1	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hepatic quanylate cyclase was further purified and characterized. The most highly purified preparation had a specific activity of 0.9 to 1.3 μmol/mg-min, S_{20w} of 6.38, Stokes radius of 47.1 Å, and molecular weight of 133,000 daltons with a monomer molecular weight of 69,000 daltons. The products of the quanylate cyclase reaction, cGMP and PP_i were definitively identified and quantitated by ^{31}P NMR. An activator of quanylate cyclase from rat liver was further purified and characterized.

276

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To purify guanylate cyclase and elucidate the mechanisms that regulate its activity.

Methods Employed: Guanylate cyclase is assayed in a system containing 1 mM [α-^{32}P]GTP, 5 mM $MnCl_2$, 1 mM cGMP, 6 mM theophylline, 50 μg of activator, and Tris-Cl buffer, pH 7.4. [^{32}P]cGMP was purified through columns for radioassay. The cyclase from supernatant of tissue homogenates is purified using salt fractionation, ion exchange, gel filtration, affinity chromatography, and electrophoresis.

Major Findings: Purification and Characterization of Hepatic Guanylate Cyclase. ^{31}P NMR (nuclear magnetic resonance) has been utilized to modify the purification procedure of guanylate cyclase from rat liver as well as to identify and quantify the products of the reaction, cGMP and inorganic pyrophosphate. Incubation conditions different from those in the usual assay were required. Since it was necessary to accumulate high concentrations of products, high concentrations of enzyme and substrate were utilized. Because of the paramagnetic effect of Mn^{++}, Mg^{++} was substituted; this reduced the reaction rate by 80-90%. Theophylline, which inhibited purified guanylate cyclase, activator fraction, which only slightly increased activity at high enzyme concentrations, and the cGMP "trapping pool" were omitted.

Purification procedures were as previously reported (Tsai et al., J. Biol. Chem. 253: 8452, 1978) through $(NH_4)_2SO_4$ precipitation, chromatography on DEAE-Biogel and phenyl-Sepharose. After chromatography on Ultro-Gel AcA34, guanylate cyclase was applied to a column of GTP-Agarose. A small amount of phosphodiesterase was removed with 0.1 N NaCl; guanylate cyclase (specific activity 0.3-0.5 μmol/mg/min) was eluted with 0.3 M NaCl.

During incubation of guanylate cyclase from Agarose-GTP for 16 hr, NMR spectra showed a progressive increase in signal of ^{31}P cGMP as well as $^{31}PP_i$ and ^{31}P-phosphorus and a decrease in signals of Pα, Pβ and PγGTP. After 46 hr, 20 min of incubation, it was calculated that the reaction mixture contained 0.48 mM GTP, 1.56 mM cGMP, 1.18 mM PP_i, and 1.10 mM P_i. There were no signals of Pα, Pβ GDP, or ^{31}P 5'-GMP. This demonstrated that the enzyme was free of nucleotidase (GDPase) and phosphodiesterase but contained pyrophosphatase. Therefore, the enzyme was subjected to preparative polyacrylamide gel electrophoresis in 200 mM glycine buffer, pH 7.8, to remove pyrophosphatase and other contaminated proteins. With cyclase purified by preparative electrophoresis (specific activity 0.9-1.3 μmol/mg/min), cGMP and PP_i were the only products observed in NMR spectra. After incubation for 15 hr with 2 mM GTP, the spectrum indicated the presence of 0.71 mM cGMP and 1.28 mM GTP. This most highly purified preparation exhibited the highest specific activity reported from any mammalian tissue. This purified cyclase has a sedimentation constant (S_{20w}) of 6.38, Stokes radius of 47.1 Å, and molecular weight of 133,000. On SDS-polyacrylamide gel electrophoresis, the cyclase revealed a major stained protein band which correlated well with the amount of enzyme activity applied and a very lightly stained band. We have also purified the cyclase from calf liver with the same procedure and obtained a specific activity of 0.9 μmol/mg/min. The purified cyclase from calf liver contained the 69,000 dalton band.

The calf liver guanylate cyclase purified from GTP-Agarose was inhibited by oxidized glutathione (GssG) and p-chloromercuribenzoate (pCMB). The cyclase lost 80% of its activity at 30°C for 10 min but was partially protected by addition of 100 μg γ-globulin and completely prevented by the addition of only 12 μg of a partially purified activator from rat liver. The cyclase (in the presence of γ-globulin) was inhibited 50% by 10 mM GssG; inhibition by GssG required incubation at 30°C for 5 min. Addition of 8 mM dithiothreitol returned the activity to control level. pCMB, 10 μM, inhibited 95% of the cyclase activity, and the inhibition was completely reversed by dithiothreitol.

Activator of Guanylate Cyclase. Guanylate cyclase activity is increased by a fraction prepared from rat liver as previously described (Tsai et al., J. Biol. Chem. 253: 8452, 1978). The activator fraction was stable to the treatment with 0.5 N NaOH at room temperature overnight. Chromatography of the NaOH-treated fraction was excluded from the column; the other (pII fraction) was eluted with approximately 200 mM NaCl solution. 10 to 300 μg of pII maximally activated the cyclase. Chromatography of the pII fraction on BioGel-A 1.5 column yielded 2 protein peaks which increased guanylate cyclase activity; one was excluded in the void volume and the other was retained in the column. After incubation of the pII fraction with 1% SDS and 5% mercaptoethanol at 100°C for 20 min and chromatography on Sephacryl S-300 equilibrated and eluted in the absence of SDS, a single protein peak with a molecular weight of 150,000 daltons was obtained. The activation of the cyclase was coincident with this protein profile. The activator was capable of activating guanylate cyclase purified from both rat and calf livers.

Significance to Biomedical Research: Although at present information concerning the metabolism and functions of cyclic GMP is limited, it appears that this nucleotide may be of special importance in the development, physiology, and pathology of lung, vascular smooth muscle, and kidney.

Proposed Course: We shall further characterize the properties of pure guanylate cyclase and also purify the activators in order to study their interaction with and mechanisms of activation of guanylate cyclase.

Publications: Tsai, S.-C., Shindo, H., Manganiello, V.C., Adamik, R., and Vaughan, M.: Products of reaction catalyzed by purified rat liver guanylate cyclase determined by ^{31}P NMR spectroscopy. Proc. Natl. Acad. Sci. U.S.A., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00614-03 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase from Bovine Brain: Purification and Characterization

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Randall L. Kincaid	Staff Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Ellis S. Kempner, Laboratory of Physical Biology, NIAMDD, NIH.

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain has been purified by two methods, yielding enzymes with identical specific activities. SDS gel electrophoresis of the purified enzyme yields a single major polypeptide of 58,000 daltons. The catalytic properties of the enzyme in the presence of calmodulin, polyamines, and metal ions have been examined. The use of pH gradient chromatography on ion exchange gel and electrofocusing of the purified enzyme indicate (at least) two calmodulin-stimulated isoenzymes. Limited proteolysis of the purified enzyme yields a catalytically active peptide of ∿ 40,000 daltons which does not interact with a calmodulin-Sepharose affinity gel.

Project Description:

Objectives: To purify the calmodulin-dependent phosphodiesterase from bovine brain and characterize the enzymatic and physical properties of the protein(s). To investigate the interaction of the calmodulin-binding regions of the enzyme after limited proteolysis and to examine the molecular basis of the apparent chromatographic and electrophoretic heterogeneity in the purified enzyme activity.

Methods Employed: Two methods have been used to purify the enzyme to ∿ 70% homogeneity. In Method I, supernatant is fractionated by use of pH gradient chromatography on anion-exchange cellulose or gels. The fraction eluting between pH 5.8 and 5.3 is pooled and further purified by gel filtration on Sephacryl S-200 and/or hydrophobic chromatography on phenyl or hexyl-Sepharose. The active fraction is then applied to an affinity gel of calmodulin-Sepharose and eluted with EGTA. Method II utilizes calcium phosphate gel adsorption of supernatant activity followed by repeated gel filtration and affinity chromatography.

The method of "target theory" analysis has been used to estimate the molecular size of the catalytic moiety. In this method, samples frozen at -150°C were subjected for varying periods of time to high energy ionizing irradiation. The loss of activity as a function of irradiation can be used to estimate the apparent size of the protein.

Major Findings: The calmodulin-sensitive enzyme purified by either of the two methods yields an activity 3,600- to 4,200-fold purified over supernatant, with a peptide of 58,000 daltons being the major component (∿ 70%) stained on SDS gels. The functional size of the catalytic moiety determined by target theory analysis corresponds to 60,000 daltons, irrespective of whether purified enzyme, gel filtration fractions, or crude supernatant was irradiated. The same value was observed when gel filtration fractions of M_r range 60,000 to 70,000 daltons or 120,000 to 150,000 daltons were used. Taken together, these data suggest the active enzyme unit is a monomer, although higher associated forms of the enzyme may be present.

The enzyme purified by Method I appears to be several closely related isoenzymes. When the enzymes are electrofocused, two major activity peaks are observed with isoelectric points of 5.45-5.50 and 5.60-5.65. The enzyme activity is stimulated 3- to 5-fold by Ca^{++} and calmodulin with an apparent half-maximal stimulation at 5×10^{-10} M calmodulin. Limited proteolysis of the enzyme by α-chymotrypsin yields a major polypeptide of ∿ 40,000 daltons which is catalytically active. The protein is not stimulated by calmodulin, nor can it bind to calmodulin-Sepharose, suggesting the calmodulin-sensitive domain has been cleaved.

The enzyme purified by Method II exhibits a specific activity identical to that prepared by Method I (40-50 μmol cGMP hydrolyzed per min/mg protein at 30°C) but is stimulated 6- to 10-fold in the presence of calmodulin.

Findings not Directly Related to Purification of the Enzyme: The purification method introduced previously, sequential adsorption-electrophoresis, has been carried out successfully with enzyme bound to calcium phosphate gel and with phenyl-Sepharose, indicating the general utility of this method for separation of proteins bound to adsorbents or affinity matrices.

Significance to Biomedical Research: Many biochemical effectors, such as hormones, are known to produce changes in the activity of mammalian cells by altering the rate of synthesis and/or degradation of cyclic nucleotides. The present study of the enzyme involved in the regulation of the processes is important for understanding the normal and pathologic cellular activity and will permit design of rational therapeutic approaches. The increasing significance of calmodulin-mediated events in diverse cellular functions makes the attempt to define the molecular domain and mechanism of action of this mediator of Ca^{++}-dependent effects important.

Publication: Kincaid, R.L. and Vaughan, M.: Sequential adsorption-electrophoresis: Combined procedure for purification of calcium-dependent cyclic nucleotide phosphodiesterase. Proc. Natl. Acad. Sci. U.S.A. 76: 4903-4907, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00615-03 CM

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Production and Characterization of Monoclonal Antibodies Against Bovine Brain cGMP Phosphodiesterase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mary Ann Danello	Staff Fellow	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
	Vincent C. Manganiello	Head, Section on Biochemical Physiology	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cellular Metabolism

SECTION

Biochemical Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.4	1.3	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Current work is focused on purification and regulation of cGMP phosphodiesterase in bovine brain. The cGMP phosphodiesterase activity being studied is activated in the presence of calcium and calmodulin and may be modulated by other factors as well. Recent advances in the production of monoclonal antibodies, using specialized cell fusion techniques, permit the isolation and characterization of extremely specific antibodies to complex antigens. Such antibodies made in response to highly purified phosphodiesterase preparations are useful for pursuing various investigations including enzyme localization and regulation on the cellular level.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The activities of cyclic nucleotide phosphodiesterases in mammalian tissues are under stringent regulatory control. Studies concerning such regulation as well as those probing tissue specificity and localization of phosphodiesterase are facilitated with the use of a purified enzyme preparation. Because of the specific interaction of antibodies with antigens, antibodies are frequently used as tools for enhancing purification procedures as well as for investigating interactions of proteins on a molecular level. Current use of hybridoma cells (hybrids of T or B cells with a continuous myeloma cell line), which produce monoclonal antibodies, enables one to select and propagate antibodies against specific antigenic determinants, even from a partially purified antigen.

The objective of this work is to isolate and characterize monoclonal antibodies made in response to a highly purified preparation of calmodulin-dependent cGMP phosphodiesterase from bovine brain. Such antibodies can then be utilized to investigate the nature of the interactions of phosphodiesterase with other proteins which modulate its activity. Additionally, studies concerning cellular localization and distribution of the enzyme can be pursued.

Methods Employed: Highly purified isolates of bovine brain cGMP phosphodiesterase were prepared by Dr. Randall Kincaid. One preparation (≈ 600X purified) consisted of enzyme activity eluted from sequential adsorption electrophoresis of bovine brain crude supernatant (Kincaid and Vaughan, 1979). A second preparation (≈ 3000-4000X purified) was obtained in the following manner: crude brain supernatant was adsorbed to and eluted from calcium phosphate gel, repeatedly chromatographed on Ultrogel AcA44 and finally eluted from calmodulin-Sepharose. Both enzyme preparations were used as antigens in the production of monoclonal antibodies.

Monoclonal antibodies were prepared from Balb C mice as described by Kohler and Milstein (1975) and detected using a solid phase indirect radioassay. Polyvinyl, V-shaped, 96-well plates were coated with purified rabbit anti-mouse IgG. Hybridoma supernatants containing antibodies were then allowed to incubate in the wells followed by the addition of ^{125}I-phosphodiesterase. Specific binding of ^{125}I-phosphodiesterase to the wells was quantitated in a gamma counter, and hybrids producing anti-phosphodiesterase antibody were frozen for subsequent cloning and propagation.

Major Findings: Injection of Balb C mice with highly purified preparations of cGMP phosphodiesterase from bovine brain resulted in the production of mouse spleen cells capable of hybridizing with mouse myeloma cells (i.e., theoretically, antibody-producing cells). To date, screening of such hybrids for antibody specific for phosphodiesterase has been negative, although antibodies to other proteins in the preparation might be present.

Proposed Course: To increase the antigenic capabilities of the enzyme preparation, an in vitro immunization procedure will be attempted (Luben and

Mohler, 1980). Once the antibodies are obtained, they will be characterized and then utilized in a variety of physical and chemical studies with the phosphodiesterase.

Significance to Biomedical Research: Production of monoclonal antibodies to a major regulatory enzyme in mammalian cells is of importance for understanding not only the nature of the enzyme itself but also for elucidating the processes which regulate its activity within the cells.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00617-04 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Mechanism and Action of Anti-Inflammatory Drugs; Effects on Neutrophil Mobilization in Carrageenan- and Dextran-Induced Inflammation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alvair P. Almeida	Guest Worker	CM	NHLBI
	Theresa N. Lo	Research Chemist	CM	NHLBI
OTHERS:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI
	Barbara M. Bayer	Staff Fellow	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Almeida is an International Research Fellow, Fogarty International Center, NIH.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	1.5	0.2

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Carrageenan injected into the rat pleural cavity induces after a short delay extravasation of serum protein, the appearance of neutrophil chemotactic factor(s) and, after 90 min, the infiltration of large numbers of neutrophils (80-140 x 10^6 cells/cavity). Pleural mast cells remained intact. Indomethacin given orally or intravenously aborted these reactions whether given before or after neutrophil infiltration started but, unlike other immunosuppressive drugs, did not affect production or release of white cells from bone marrow. The intrapleural injection of low molecular weight Dextrans (T10) provoked a similar reaction, but high molecular weight Dextrans (T70) promoted mast cell degranulation, extensive edema and little (< 30 x 10^6 cells) neutrophil infiltration. Intermediate molecular weight Dextrans (T40 and T70) produced both reactions. The studies indicated that mast cell degranulation and neutrophil accumulation are distinct reactions, although both may occur simultaneously in some forms of experimentally induced inflammation. Indomethacin inhibits specifically the neutrophil response.

Project No. Z01 HL 00617-04 CM

Project Description:

Objectives: Carrageenan-induced pleurisy in rat was chosen as a model to study the mechanism of action of the anti-inflammatory drugs. Studies by other workers (see Fed. Proc. 35: 2447, 1977) have shown that carrageenan injected into the pleural cavity will induce an accumulation of large numbers of white cells (predominantly neutrophils and, at later stages, monocytes). The number of cells mobilized is suppressed by the anti-inflammatory drugs. Similar responses are evoked by other macromolecules, such as dextran and glycogen, although some differences have been noted in the time course of response and the extent of early edema (fluid) and neutrophil accumulation. In this program, the release of inflammatory mediators as well as chemotactic factors will be investigated. We shall attempt also to identify the source of white cells and factors responsible for their various forms of activity, e.g., recruitment, chemotaxis and phagocytosis, and the effects of anti-inflammatory drugs on the processes.

Last year's studies showed that after carrageenan there is no degranulation of mast cells (or histamine release) and little accumulation of fluid in the pleural cavity but, after a delay of 90 min, a rapid increase in the number of neutrophils. By 4-8 hr, as many as 80-140 x 10^6 cells had accumulated in the cavity. These cells were derived from the bone marrow. Prolonged (5 days) treatment with the immunosuppressant drug, methotrexate, was necessary before the response to carrageenan was curtailed. Indomethacin, in contrast, prevented the accumulation of white cells in the pleural cavity without affecting the production, storage, or release of white cells in the bone marrow. The present work is a continuation of these studies.

Methods Employed: Inflammation was induced in Sprague-Dawley rats (200 g) by injection of 500 µg carrageenan or 6 mg of the dextrans into the pleural cavity. Rats were killed at the indicated times and the pleural fluid collected. Volume of the exudate and cell counts were determined as described previously. Differential cell counts were determined by use of the Cytocentrifuge and differential staining of the slides. Blood cell counts were obtained from air-dried smears of blood taken by cardiac puncture. Anti-inflammatory drugs were administered orally as suspensions in cornstarch gel or by intravenous injection. Other drugs and labeled compounds were injected as saline solutions by the routes indicated. Histamine and serotonin were assayed by the enzymatic isotopic techniques described in previous annual reports.

Changes in vascular permeability were assessed by i.v. injection of 0.5 ml of a 2% w/v Evans blue solution immediately after the intrapleural injection of the inflammatory agent. The supernatant fraction of the inflammatory exudate was assayed for the dye by measurement of optical density (O.D.) at 600 nm. Control animals received saline by intrapleural injection.

Chemotactic activity of various exudate fractions was measured in modified Boyden chambers. Partially purified (80-85%) suspensions of neutrophils were prepared by standard techniques from blood of rats which had been injected intrapleurally with carrageenan 4 hr previously and used as the source of test cells.

Major Findings: Time Course of Response to Carrageenan. Within 30 min, small amounts of a fluid (0.1-0.2 ml) had collected in the pleural cavity. This fluid contained small amounts of dye and chemotactic factor(s). After 75 min, increasing numbers of neutrophils appeared in the exudate and by 4 hr the exudate (1-2 ml) consisted almost entirely of neutrophils ($< 95\%$). The peak response occurred at about 8 hr, at which time monocytes were also observed in the exudate. Thereafter, the volume and numbers of cells decreased. Although there was a modest increase in the numbers of circulating neutrophils (27-65%), the total number of neutrophils in the chest cavity far exceeded that in the circulation. The amount of dye in the cavity increased progressively during the course of the reaction.

Dextran-Induced Edema. Differences in response were noted with different molecular weight Dextrans. With a low molecular weight, Dextran (T10), the response was similar to that observed with carrageenan. There was little degranulation of mast cells or release of histamine and little accumulation of fluid until 2-4 hr, when extensive infiltration of neutrophils ($\sim 80 \times 10^6$ cells/cavity) was evident. With a higher molecular weight, Dextran (T70), there was extensive degranulation of mast cells, the appearance of free histamine in the pleural exudate and infiltration of a relatively small number of neutrophils ($\sim 30 \times 10^6$ cells/cavity). Dextrans of intermediate molecular weight (T40 and T70) produced both responses, but the extent of histamine release or neutrophil recruitment was less than that produced by T70 or T10, respectively.

The Effect of Anti-Inflammatory Drugs. In all cases, the anti-inflammatory drugs suppressed neutrophil accumulation. With the carrageenan model, the extent of suppression was related to the dose of drug and the potency of the drug (ED_{50}) was similar to that reported in other test models of inflammation, i.e., indomethacin (ED_{50}, 2.5 mg/kg) was more potent than phenylbutazone (100 mg/kg) or aspirin (ED_{50}, 150 mg/kg). Indomethacin (1 mg/kg, i.v.) aborted the reaction whether given prior to or immediately after neutrophil accumulation had started.

Indomethacin did not suppress mast cell degranulation, histamine release, or the early fluid accumulation.

Studies on the Interaction of White Cells and Heparin. Since heparin is released upon degranulation of mast cells, its interaction with the cellular infiltrates was investigated. Heparin was found to induce gel formation not only with suspensions of neutrophils but also of white cells isolated from bone marrow, spleen and thymus. The tenacity of the gel was related to heparin concentration (in the range 5-220 units/ml) and to the number of cells (5 to 60×10^6 cells/ml) in suspension. Although the cells remained uniformly dispersed in the gel (aggregation or flocculation was not evident), they did not readily sediment upon centrifugation.

Gel formation occurred only with intact cells (supernatant extracts did not gel) and when temperatures of the suspensions were above 20°C. The reaction differed from that described for heparin-induced platelet aggregation. The reaction was not induced by ADP nor was it Ca^{++}-dependent or inhibited by aminoacrine and prostaglandin synthetase inhibitors. It was inhibited, however, by protamine and incubation with agents which led to loss of cell viability, e.g., deoxyglucose or antimycin A. Estimates of the heparin content (20 pg/mast cell) indicated that the amount of heparin released into the pleural cavity by histamine releasers, such as Dextran T70, would be sufficient to induce increased white cell stickiness or gel formation.

Significance to Biomedical Research and the Program of the Institute: Carrageenan- and Dextran-induced edema appear to be useful models to study the effects of various drugs on different types of inflammatory reactions. With these models we have shown that 1) mast cell degranulation (and early edema formation) and neutrophil accumulation are unrelated phenomena, although both may occur in response to a single agent, 2) high molecular weight Dextrans evoke mainly the former reaction and low molecular weight Dextran and carrageenan the latter, 3) increased vascular permeability and production of chemotactic factors are evident before neutrophil accumulation, and 4) indomethacin and other nonsteroidal anti-inflammatory drugs inhibit the recruitment of white cells but not the edema induced by mast cell degranulation.

Proposed Course: Subsequent studies will investigate whether the suppression of neutrophil accumulation by the anti-inflammatory drugs is due to inhibition of production of chemotactic factor(s) or the ability of the cells to respond to chemotactic factors.

Publications: Horakova, Z., Bayer, B.M., Almeida, A.P., and Beaven, M.A.: Evidence that histamine does not participate in carrageenan-induced pleurisy in rat. Eur. J. Pharmacol. 62: 17-25, 1980.

Almeida, A.P. and Beaven, M.A.: Gel formation with leucocytes and heparin. Life Sci. 26: 549-555, 1980.

Almeida, A.P., Bayer, B.M., Horakova, Z., and Beaven, M.A.: Influence of indomethacin and other anti-inflammatory drugs on mobilization and production of neutrophils: Studies with carrageenan-induced inflammation in rats. J. Pharmacol. Exp. Ther., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 00619-04 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Histamine in Gastric Secretion. Studies with Isolated Gastric Mucosal Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI

COOPERATING UNITS (if any)
Drs. Andrew H. Soll and Morton I. Grossman, Center for Ulcer Research and Education, Wadsworth V. A. Hospital, Los Angeles, California

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.3	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies with highly purified preparations of cells from collagenase digests of dog gastric mucosa indicate that histamine (and histidine decarboxylase) is located in mast cells (histamine content 2.5 pg/cell) which reside in the lamina propria in close proximity to the parietal cell. The parietal cells contain most of the high histamine methyltransferase activity found in the mucosa, and this may account for the absence of free histamine in gastric seretion or gastric venous blood in this species. In contrast, in rat, histamine is loated in an enterochromaffin-like (ECL) cell which contains abundant histamine (2-4 pg/cell), histidine and DOPA decarboxylase activity, and has the properties of APUD cells. These cells do not possess IgE receptors and lie in the base of the gastric glands at some distance from the parietal cell. The activities of the histamine-inactivating enzymes are low in rat, and, unlike dog, histamine appears in rat urine during periods of gastric secretion. Unfinished studies indicate that the histamine-containing cells after short-term (24 hr) culture release histamine in response to some, but not all, of the histamine-releasing agents.

Project Description:

Objectives: The finding that the histamine H_2 receptor antagonists block gastric secretion in a variety of species provided irrefutable evidence that histamine has an essential role in gastric secretion. Histamine's relationship to other mediators of gastric secretion, gastrin and acetylcholine is still, however, poorly understood, nor is it known how histamine is released or how it reaches the acid-secreting parietal cell. The successful separation and identification of the different cell populations in dog gastric mucosa in collaboration with Soll and Grossman at the Center for Ulcer Research and Education (CURE), Wadsworth V. A. Hospital, Los Angeles, has given us an opportunity to answer some of these questions. This technique led to the identification of the "histamine-containing cell" in the gastric mucosa of dog and to obtain preliminary data as to its identity in rat gastric mucosa. In this report, we described studies which compare and contrast histamine-containing cells in dog and rat mucosa with those in dog liver and rat peritoneum and which have allowed us to identify the sources of the enzymes responsible for the synthesis and inactivation of histamine.

Methods Employed: Gastric mucosa and liver were treated sequentially with a collagenase preparation, EDTA, and then a second time with collagenase. The cells were harvested from the disrupted mucosa and then separated in a Sorvall zonal rotor through an albumin density gradient. This procedure separated the cells into histamine, parietal, and chief cell-enriched fractions. Each of these was further fractionated according to cell size with the Beckman elutriator. Gland preparations (isolated glandular crypts) were prepared by partial digestion with collagenase and centrifugation through Ficoll solution. Rat peritoneal mast cells were fractionated by elutriation. At all stages, cell viability was monitored by microscopy and dye exclusion tests. The fractions were divided into portions of about 10^6 cells, and duplicate sets were shipped from CURE to the NIH.

At the NIH, the fractions were assayed for DNA, histamine and serotonin content, as well as for histidine decarboxylase, DOPA decarboxylase, histamine-N-methyltransferase (HNMT) and diamine oxidase activities, using the microassays developed in this laboratory. These assays have been described in previous annual reports, although further refinement of some procedures was necessary because of the small size of the samples. Assays were also performed by the principal investigator on fresh samples at CURE.

IgE binding was studied by procedures devised by Dr. Henry Metzger (NIAMDD), who supplied ^{125}I-labeled rat myeloma protein for these studies.

Major Findings: Isolation and Properties of the Histamine-Containing Cells in Dog Liver and Fundic Mucosa. Profiles obtained by separation of different cell fractions first by elutriation and then by density gradient separations produced a single sharp peak of histamine for both dog liver and mucosa. In both tissues, the highest histamine concentration was found in cells with a mean density of 1.080 and size of 11 µm. Mast cell constituted more than 80% of the cells in these fractions. The average histamine content was 2.5 ± 0.3 pg/cell ($n = 3$) for dog mucosa and 2.1 pg/cell for liver (Table). There was a close correlation ($r = 0.94$) between mast cell count and histamine content in both tissues. Electron micrographs confirmed that a high proportion of the cells possessed the typical morphology of mast cells with characteristic dense granular structure. More than 90% of the cells were viable, as indicated by trypan blue exclusion.

Measurement of DNA as well as histamine at all stages of purification indicated that the histamine content of the unfractionated mucosal cells represented 69% of the histamine content in intact mucosa and that less than 20% of the histamine was lost through subsequent purification steps. On the assumption that each mast cell contained 2.5 or 2.1 pg histamine, it was calculated that the mucosa contained 3×10^7 mast cells/g, or about one mast cell per 3 parietal cells, and that liver contained 1×10^7 mast cells/g tissue.

In morphological studies of intact mucosa, the mast cells were found in the lamina propria along the basement membrane of the gastric glands. Most of these cells were in close proximity to parietal cells. By light and electron microscopy, we failed to find mast cells on the glandular side of the basement membrane of the epithelium. Preparations of gastric glands, which were separated from the basement membrane and lamina propria by the enzyme treatment, contained less than 20% of the histamine found in either the whole mucosa or unfractionated mucosal cell suspension.

Histidine decarboxylase activity was found only in the mast cell fractions of the mucosa and liver cell preparations, but in the mucosal cells the activity was low compared to that in the liver or rat peritoneal mast cell preparations (Table).

Distribution of Histamine, Serotonin and Enzyme Activity in Dog Gastric Mucosa. Mucous-, chief-, parietal-, histamine- and serotonin-containing cells could be clearly identified in different regions of the profiles obtained by density gradient and elutriator separation. Serotonin co-separated with enterochromaffin cells which appeared to contain glucagon and DOPA decarboxylase activity. These cells have not been further characterized. Histamine-N-methyltransferase activity was located largely in the parietal cell region (correlation coefficient for parietal cells and enzyme activity, $r = 0.85$, $p < 0.005$), although a smaller fraction (~ 30%) was located in smaller cells which remain to be identified. Diamine oxidase activity was not detectable in intact mucosa or any of the different cell fractions.

Separation of Histamine- and Serotonin-Containing Cells in Rat Gastric Mucosa and Peritoneum. The histamine-containing cell in the rat mucosa was quite different from that in dog mucosa. It lacked the typical granular appearance of the mast cell and had the staining characteristic of an enterochromaffin-like (ECL) cell described by other authors (Thunberg, Exp. Cell Res. 47: 108-115, 1967; Hakanson *et al.*, J. Pharmacol. Exp. Ther., 1974). These cells, unlike the dog mucosal mast cells, lay inside the gastric gland alongside the basement membrane but somewhat away from the parietal cells.

The fractions of cells with the highest histamine content contained approximately 18% of the ECL cells and a histamine content of 2-4 pg/cell (n = 3).

No serotonin was found in these cells (Table). The cells were highly vacuolated but were devoid of electron dense granules. In contrast, rat peritoneal cells contained 14 pg histamine and 0.5 pg serotonin/cell and possessed numerous dense metachromatic granules.

The profiles of other biochemical markers also differed to that seen with dog mucosa. The ECL cells, in addition to histamine, contained high histidine decarboxylase activity and no serotonin. No histamine methyltransferase activity was detected in any cell fraction. Diamine oxidase activity was present in a fraction of large cells, but the profile of activity did not fit that for parietal cells.

Histamine Release from Histamine-Containing Cells from Gastric Mucosa and other Tissues: Presence of IgE Receptors. As reported by Metzger and associates, rat peritoneal cells contained an abundant number of IgE receptors (247,000 ± 60,000/cell, n = 6). None were found on the rat gastric mucosal ECL cell, and the rat IgE did not cross-react with the receptors in the dog liver or gastric mucosa mast cells.

Although rat peritoneal mast cells were fully responsive to Compound 48/80, Na^+ ionophore, and various histamine-releasing peptides, these agents did not release histamine from freshly isolated rat ECL cells or mast cells from dog liver and gastric mucosa. Additional maneuvers, such as the addition of phosphatidyl serine and albumin or use of other media, were ineffective. Short-term culture (24-48 hr) of the isolated cells led to some responsiveness (up to 25% release) of the cells to ionophore, the histamine-releasing peptides, and, with dog cells, ascaris antigen. Release in response to gastrin and carbachol was variable. Further studies with the short-term cultures are required before we can assess the significance of these findings.

Additional Characteristics of the Rat Gastric Mucosal ECL Cell. As reported for the ECL in intact tissue, the isolated cell did not exhibit chromaffin staining but did possess APUD characteristics as defined by A. G. E. Pearse; that is, the cells were able to take up and decarboxylate dihydroxyphenylalanine (or 5-hydroxytryptophane) and acquire chromaffin properties. In one experiment, the cells did not stain after incubation with a rat myeloma IgE antibody which was labeled with rhodamine.

Conclusions and Significance to Biomedical Research: The studies reveal differences in the architecture of the gastric glands and their association with the histaminocytes. In dog, the distance between mast cells and target receptor (see previous reports for discussion of receptors on parietal cells) is small. Presence of high histamine methyltransferase activity on or in the parietal cell probably ensures that little free histamine escapes into the gastric circulation. This is consistent with the failure of many experienced workers over the past 40 years to demonstrate histamine release from dog stomach. In rat, histamine-inactivating activity is weak and histamine must diffuse some distance, either across extracellular spaces or into the small blood vessels of the lamina propria, before reaching the parietal cell. Unlike other species, histamine levels increase in rat urine during periods of gastric stimulation. Although the present studies begin to provide a picture of histamine's location and, upon release, its disposition and access to receptors, we are still uncertain as to the mechanism(s) of its release.

Proposed Course: Future studies will focus on possible mechanisms of histamine release from histamine cells by use of short-term culture experiments, particularly their responsiveness to gastrin and cholinergic agents. More detailed biochemical examination of these cells (e.g., activation of adenylate cyclase, Ca^{++} flux) will follow once the agents inducing histamine release have been identified.

Publications: Soll, A.H., Lewin, K., and Beaven, M.A.: Isolation of histamine containing cells from canine fundic mucosa. Gastroenterology 7: 1283-1290, 1979.

Beaven, M.A.: Factors Regulating Availability of Histamine at Tissue Receptors. In Ganellin, C.R. and Parsons, J. Eds.): Pharmacology of Histamine Receptors. London, John Wright and Sons, Ltd., in press.

TABLE

Characteristics of isolated histamine-containing cells in different species

Preparation	(n)	Morphology	Histamine content	Serotonin content	DOPA decarboxylase	Histidine activity
			(pg/cell)		(pmoles/10^6 cells/hr)	
Dog						
Gastric mucosa	(3)	Mast cell	2.5 ± 0.3	< 0.1	< 100	12,17
Liver	(2)	Mast cell	1.9, 2.2	---*	--	51,48
Rat						
Gastric mucosa	(3)	ECL-like cell	2-6	< 0.1	2,700, 1,300	145 ± 30
Peritoneal cells	(5)	Mast cell	14 ± 3	0.5 ± 0.1	< 100	30 ± 8
Blood basophils**		Basophil	1.1	---	--	---
Man						
Blood basophils**		Basophil	1.1	---	--	low

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* Not determined

** As reported by Graham et al.

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PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Histamine Synthesis, Release and Metabolism in Tissues

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI
	Barbara M. Bayer	Staff Fellow	CM	NHLBI
OTHER:	Alvair P. Almeida	Guest Worker	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Almeida is an International Research Fellow, Fogarty International Center, NIH. Dr. Jonathan Moss, Dept. of Anesthesiology, Massachusetts General Hospital, Boston. Dr. Andrew Soll, Wadsworth V. A. Hospital, Los Angeles.

LAB/BRANCH

Cellular Metabolism

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.3	0.6

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Histidine decarboxylase activity of isolated intact mast cells from rat peritoneum, dog gastric mucosa and dog liver was 10-20 times greater than that of disrupted cells or soluble extracts of these cells. Measurement of histidine uptake and the effects of amino acids that compete with this uptake suggested that histidine transport may contribute to the high histamine synthetic activity of the intact cells. Histamine methyltransferase, which is responsible for histamine inactivation in most tissues, is inhibited by H_2 histamine receptor agonists of which impromidine is the most potent inhibitor (K_i, 10^{-7} M). This compound is also a potent inhibitor of diamine oxidase (K_i, 10^{-8} M). The work continues to indicate the diversity of cells storing histamine in the body. In addition to mast cells, brain neurones and rat gastric ECl cells (see other annual reports), high histamine levels were found in dividing human fibroblasts. In blood, the basophil is a major repository for histamine in most species, but in pig and rabbit the platelet was found to be the major source of histamine.

Project Description:

Objectives: Our studies on the source of histamine in gastric mucosa and brain raised several questions related to histamine metabolism and release during pathological/physiological reactions. The histidine decarboxylase activity was insufficient to account for the histamine levels in these tissues. The high histamine methyltransferase (HMT) activity in the parietal cell in dog mucosa also suggested that inhibitors of HMT would be particularly useful in studies of the role of histamine in gastric secretion. Another question was concerned with the relationship between plasma histamine levels and the responses induced in the whole animal.

Three studies were undertaken to address these questions, namely: 1) measurement of histamine synthesis in intact cell systems, 2) studies with putative inhibitors of HMT, and 3) measurement of plasma histamine levels in various species under normal conditions and after induction of histamine release by injurious or anaphylactic reactions. For these studies, we utilized freshly isolated mast cells from rat peritoneum, human fibroblasts in culture and plasma samples from a variety of animal species and humans undergoing elective hip surgery who showed pronounced anaphylactic-type reactions to drugs.

Methods Employed: Blood Samples. Blood specimens were collected from laboratory and farm animals obtained through normal NIH sources and the farm in Poolesville, Md. Human blood was obtained from patients undergoing surgery for hip replacement at the Harvard Medical School. These patients were monitored continuously for heart rate, cardiac output, arterial pressure and central venous pressure, and were catherterized for collection of central venous and arterial blood. These patients had reported previous "allergic reactions" to drugs. The blood samples were assayed for blood gases and electrolytes, as well as plasma histamine, catecholamines and renin activity. All blood specimens were collected into heparinized Vacuocontainers and placed in ice. A small amount of the whole blood was withdrawn by syringe, and the remainder was centrifuged at 500 g for 15 min. The supernatant fraction was collected (platelet-rich plasma) and recentrifuged at 1500 g for 30 min (plasma).

Histamine-N-methyltransferase (HMT), diamine oxidase (DAO) and histidine decarboxylase were prepared from soluble extracts of rat kidney, ileum and stomach, respectively, by ammonium sulfate fractionation procedures. Enzyme activity was assayed by isotopic microassays as described in previous reports. For kinetic studies, the concentration of substrate was varied by the addition of unlabeled compound. To determine the "apparent" K_i value for the inhibitors, Dixon plots were analyzed by computer.

Intact mast cells were obtained from male, Sprague-Dawley rats (180 to 300 g). After decapitation, 10 ml of Hanks balanced salt solution was injected into the abdominal cavity and a peritoneal cell suspension was obtained by standard procedures. The cells were fractionated by elutriation on a Beckman elutriator rotor. Human fibroblasts were grown in cultures as described in another annual report. Cell counts and size analysis were performed using a Particle Data Counter.

For the assay of histidine decarboxylase activity in intact cells, cell suspensions were centrifuged at 100 g for 10 min and the supernatant fluid removed by aspiration. The cell pellets were resuspended in Gey's salt solution containing HEPES buffer (10 mM, pH 7.4) to give 10^6 cells/100 µl. The specificity of the assay was verified by incubating the samples with β-^{3}H-(side chain carbon)-L-histidine (20 nCi) as described previously. After trapping the $^{14}CO_2$ in Hyamine, the reaction mixture was removed and assayed for ^{3}H-histamine by isotope dilution derivative analysis. For these assays, the concentration of amino acid was 1.25 x 10^{-4} M, unless noted otherwise.

Uptake of β-^{3}H-L-histidine was measured by incubation of 10^6 mast cells with 100 nCi (10 pmoles) β-^{3}H-L-histidine in 100 µl Gey's (+ 50 mM HEPES, pH 7.4) medium for 10 min at 37°C and, in separate samples, at 0°C. The cells were deposited by centrifuging the samples through dibutylphthalate in a Microfuge (Beckman Instrument Corp.) for 1 min. The difference in ^{3}H content of the pellet incubated at 37°C with that incubated at 0°C was designated "uptake." The percent decrease of the "uptake" in the presence of drugs and amino acid was calculated.

Major Findings: 1) Histidine Uptake and Histamine Synthesis in the Intact Cells. Separation of rat peritoneal cells into different fractions by elutriation yielded a single peak of histamine in which mast cells comprised 80 to 98% of the cells. The histamine content of these cells (mean ± SEM) was 14 ± 1.8 pg/mast cell (n = 5). The distribution of histidine decarboxylase activity paralleled closely that of histamine, although the rate of histamine formation by intact cells was much greater than that of soluble extracts of these cells. The magnitude of this difference was similar when histidine decarboxylase activity was measured by $^{14}CO_2$ release from ^{14}C-carboxyl-labeled β-^{3}H-histidine. The activity was attributable to specific histidine decarboxylase in that it was inhibited by α-methylDOPA and was not associated with detectable decarboxylation of L-DOPA.

The contribution of histidine transport to the histamine synthesis in the intact cell was studied by use of amino acids which compete with the uptake of L-histidine. L-tryptophan and α-methylDOPA inhibited decarboxylation as well as uptake of L-histidine in the intact cell without inhibiting the histidine decarboxylase activity of extracts. The decarboxylase inhibitors, Brocresine and α-hydrazinohistidine, selectively inhibited decarboxylase activity without interfering with L-histidine uptake.

Unexpectedly, we found that human fibroblast cultures contained high histamine levels (~ 280 ng/10^6 cells) during exponential growth but low levels (< 30 ng/10^6 cells) once they reached confluency. Only trace amounts of histamine were recovered in the medium. Other cell cultures tested did not contain measurable quantities of histamine. Earlier studies have shown that developing hypertrophic scars and keloids in humans contain elevated histamine levels and produce symptoms (itching, hyperemia) which could be attributable to histamine release (Cohen, Beaven, Keiser and Sjoerdsma, Surgical Forum, 1972). Others (Kahlson and Rosengren, Pharmacol. Rev. 48: 155, 1968) have reported that during wound healing in rat skin high decarboxylase activity is present in the wound tissue and histamine levels in urine are elevated.

2) Studies with Inhibitors of HMT. As part of a study to find more effective inhibitors of the histamine-metabolizing enzymes (see last year's annual report), we have tested several new histamine agonists and antagonists. Of the various compounds tested, the most potent inhibitors were SKF 91488 (see last year's report) and the H_2 receptor agonist, impromidine, which is now undergoing clinical trials for the evaluation of parietal cell function. This compound is a competitive inhibitor (K_i, 10^{-7} M) of HMT and DAO ($K_i \sim 10^{-8}$ M) but not of histidine decarboxylase activity. These values are similar to those of amodiaquin ($K_i \sim 0.5 \times 10^{-7}$ M) and aminoguanidine ($K_i \sim 0.5 \times 10^{-7}$ M), which are known inhibitors of HMT and DAO, respectively. The presence of two imidazole basic groups in impromidine made us suspect that, unlike other inhibitors, this agent might be a useful ligand for affinity gel chromatography of HMT (and DAO). Our initial experiments indicate that such gels have a high affinity for HMT, both for that in partially purified and crude preparations of the enzyme.

3) Correlation of Plasma Histamine Levels and Pathological/Physiological Reaction in vivo. Plasma histamine levels in man and a variety of laboratory and farm animals (12 species, n = 4-13 for each species) were less than 1 ng/ml. Mean whole blood levels ranged from 20 to 125 ng/ml.

There were, however, several exceptions. In pig and rabbit, high levels (> 1,000 ng/ml) were found in blood. Most of this histamine could be recovered in platelets, unlike in other species whose histamine is located in basophils. The presence of histamine in platelets has been described previously for rabbit but not for pig. Another exception was rat, which had "normal" blood histamine levels (49 ± 3 ng/ml) but unusually high plasma histamine levels, 15 ± 3 ng/ml (n = 10). This has been a consistent but puzzling finding in our work. Such levels in humans and other experimental animals are associated with marked hypotension and gastric hyperacidity (see later). As histamine is less potent (by 1/10th) in rat than in other species with respect to its vascular cardiac and gastric effects, rat appears to be more resistant than other species to the effects of circulating free histamine.

Our previous studies have shown that, in patients with histaminemia, plasma histamine levels of 2-5 ng/ml are associated with gastric hypersecretion and levels above 8-10 ng/ml with generalized flushing and hypotension. During one recent episode in which a patient exhibited an anaphylactic reaction to succinylcholine, we were able to monitor release of histamine as well as various other vasoactive hormones. Plasma histamine levels rose from 2.1 ng/ml to almost 40 ng/ml within 1 min of the injection of succinylcholine infusion (100 mg). This was accompanied by an immediate drop in blood pressure and after a delay of 1-2 min an acceleration in heart rate

(69 to 145 bpm). Plasma norepinephrine (750 to 2,600 pg/ml) and epinephrine (360 to 1,960 pg/ml) increased after 1-2 min. After 3 min, plasma histamine and epinephrine levels and after 9 min plasma norepinephrine levels declined. By 20 min, plasma levels of all 3 hormones were normal. Plasma renin activity increased (from 4 to 20 units/ml) at a later time (20 min). There was a significant correlation between the changes in heart rate and norepinephrine levels ($r = 0.86$, $p < 0.05$) but not with histamine, epinephrine, or renin activity. These results suggest that changes in blood pressure are correlated withplasma histamine levels but that catecholamine release (and changes in cardiac function) may be secondary reactions of histamine release.

Significance to Biomedical Research: The studies with rat peritoneal mast cells indicate that histidine transport is an important component of the histamine synthesis pathway in the intact cell. Blockade of transport may thus provide another mode of inhibition of histamine syntheses. The studies also illustrate the diversity of cell types that store histamine. These include the tissue mast cell, blood basohil, in some species blood platelet, the human fibroblast and brain neurones. The presence of histamine in the fibroblast culture is, however, especially interesting, because it points to a possible role of histamine in wound healing or fibrotic reactions.

Proposed Course: Studies of histamine synthesis will be continued with intact mast cells from dog liver and mucosa. Factors influencing histidine uptake will be studied in more detail. Fibroblast cultures will be used to study the kinetics of histamine turnover in intact cells and how this is modified by enzyme inhibitors.

Publications: Beaven, M.A. and Shaff, R.E.: Inhibition of histamine methylation *in vivo* by the Dimaprit analog, SKF Compound 91488. Agents Actions 9: 455-460, 1979.

Moss, J., Fahmy, N., Sunder, N., and Beaven, M.A.: Hormonal and hemodynamic profile of an anaphylactic reaction in man. Circ. Res., in press.

Almeida, A.P., Flye, W., Deveraux, D., Horakova, Z., and Beaven, M.A.: Distribution of histamine and histaminase (diamine oxidase) in blood of various species. Comp. Biochem. Physiol., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00621-05 CM

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Reversible Inhibition of Cell Proliferation in Culture by Anti-Inflammatory Drugs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Barbara M. Bayer	Staff Fellow	CM	NHLBI
OTHERS:	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI
	Alvair P. Almeida	Guest Worker	CM	NHLBI

COOPERATING UNITS (if any)

Dr. Almeida is an International Reearch Fellow, Fogarty International Center, NIH

LAB/BRANCH

Cellular Metabolism

SECTION

Cellular Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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SUMMARY OF WORK (200 words or less - underline keywords)

Nonsteroidal anti-inflammatory drugs arrest growth of cells in the G_1 phase of the cell cycle. Growth continues in synchrony once drug is removed. These responses are accompanied by changes in amino acid transport. Active transport by system "A" progressively diminishes and that by system "L" is enhanced; the reverse occurs after removal of drug. Kinetic studies suggest that 1) the changes are due to alteration in the number of carriers rather than in the affinity of carriers for amino acis or Na^+, 2) the decay and recovery of the transport are dependent on turnover of carriers in the plasma membrane, and 3) transport through the "A" system occurred by a "low" and a "high" affinity component; the latter was prominent during exponential growth and was selectively inhibited by indomethacin. This component was also present in freshly isolated rat thymus lymphocytes and was likewise inhibited by indomethacin whether added directly to cell suspensions or administered (10 mg/kg) prior to isolation of cells.

Project Description:

Objectives: We have shown that indomethacin and aspirin in pharmacological doses inhibit growth of a transplantable mast cell tumor (P815) and Lewis lung carcinoma in mouse (Eur. J. Pharmacol. 37: 367, 1976) and that the nonsteroidal anti-inflammatory drugs in general inhibit growth of transformed (rat hepatoma, HTC) and nontransformed (human fibroblast) cells in culture (J. Pharmacol. Exp. Ther. 202: 446, 1977). Their potency paralleled their anti-inflammatory activity, although the cytostatic activity appeared to be unrelated to their ability to inhibit prostaglandin synthesis (de Mello et al., Biochem. Pharmacol. 29: 311, 1980) or alter cellular ATP/ADP levels (Bayer et al., in press).

Last year we reported that cultures were arrested in the G_1 phase of the cell cycle and that after removal of drug there was a short delay (18-24 hr) before growth resumed. Before the onset of growth, there was a surge in DNA synthesis which peaked between 12-18 hr and prior to the increase in cell count a sharp increase in mitotic index. Autoradiography of cultures grown in the presence of labeled thymidine and cytofluorometric estimations of cellular DNA content indicated that all (> 95%) cells resumed growth in synchrony (Bayer et al., 1979). An important finding was that the progression of cells through the cell cycle was accompanied by changes in amino acid transport. Transport through the "A" system (see Methods for definition of the transport systems) decayed and that through the "L" system was enhanced. The reverse occurred when drug was removed. The changes in the "A" system were time-dependent. Transport of deoxyglucose, thymidine and uridine were unaffected.

This year's studies provide information on the mode of action of these drugs. By use of classical kinetic analysis of the effects in several cell lines, we have determined that a high affinity component of the "A" system exists during exponential growth of cultures and that in nontransformed cell lines this component decays as the cultures reach confluency. Indomethacin selectively inhibits this component by decreasing the number of functional carriers rather than by altering the affinity of the carrier for amino acids or Na^+ which is transported with the amino acid (see Methods).

Methods Employed: Incubation Procedures. Cells were grown in Eagle's medium supplemented with Earle's salts, 2 mM glutamine and 10% fetal calf serum in an atmosphere of 95% air and 5% CO_2 at 37°C. For individual experiments, cells from confluent cultures were suspended by trypsinization or by shaking the flasks and then diluted 20-fold with fresh medium. Samples (1 ml, 20-50,000 cells) of the diluted suspension were dispensed into individual wells of tissue culture cluster plates (16 mm wells).

Preparation of Thymus Lymphocytes. Suspensions (10^6 cells/ml) of thymus lymphocytes were prepared from young (140-150 g) male rats by placing the thymuses in Dulbecco's medium, cutting open the organ capsule and gently squeezing the thymus with forceps. The cell suspension was centrifuged and the pelleted cells resuspended in fresh medium.

Addition of Drugs. Samples, 100 µl or less, of a neutralized solution of the drug (in Eagle's medium) or 10 µl of a solution of indomethacin-^{14}C were added to each well at the times indicated. To remove drug, the medium was aspirated, the cultures were washed once with medium and 1 ml of fresh medium was added. All solutions were sterilized by filtration.

Measurement of Cell Counts, Cell Viability and Mitotic Index. To count cells, the cultures were washed with Ca^{++}- and Mg^{++}-free Dulbecco's medium and then incubated with a 0.025% w/v trypsin solution. Cells were counted in a Particle Data Counter or a Neubauer counting chamber. Cell viability was assessed by ability to exclude trypan blue (0.05% solution).

Measurement of ^{14}C-Indomethacin Uptake. Culture medium was removed by aspiration and the cells were washed briefly in 0.1 ml ice-cold Dulbecco's medium and then 0.1 ml 0.4 M perchloric acid. The cell residues were washed with 0.1 ml of an ethanolic solution of unlabeled indomethacin (1 mg/ml) to extract labeled indomethacin from the precipitated cell residues.

Measurement of Amino Acid Transport. Experiments were performed in a sterile hood heated to 37°C. Cultures were washed twice in Dulbecco's medium and solutions of α-aminoisobutyric acid [^{3}H] (AIB), α-methylaminoisobutyric acid [^{14}C] (MeAIB), aminobicycloheptyl-carboxylic acid [^{14}C] (BCH), or mixtures of these (20 nCi of ^{14}C- and 100 nCi of ^{3}H-labeled compounds) in Dulbecco's medium were added. The cultures were incubated for 10 min unless indicated otherwise, placed on ice, washed twice with ice-cold Dulbecco's medium (Ca^{++}- + Mg^{++}-free) and lysed with 200 µl water. Samples (100 µl) of the lysate were assayed for radioactivity. Unlabeled amino acids were added along with the labeled compounds. Other procedures were described in detail in last year's annual report.

Definition of Amino Acid Transport Systems. These systems have been defined by Christensen and co-workers in a variety of mammalian cell lines by the use of nonmetabolizable neutral amino acids, which include α-aminoisobutyric acid (AIB), α-methylaminoisobutyric acid (MeAIB) and aminobicycloheptdyl-carboxylic acid (BCH). The "A" system, which is Na^{+}- and energy-dependent, has an affinity for AIB and MeAIB and most neutral amino acids; a Na^{+}-dependent "ASC" system, which has an affinity for nonmethylated amino acids (it does not take up MeAIB); and an "L" system, which is not Na^{+}- or energy-dependent and has a high affinity for BCH and other lipophilic amino acids such as leucine. "Lysine" and "aspartate" preferring systems have also been described for the transport of basic and acidic amino acids.

Major Findings: Amino Acid Transport Systems Affected by Anti-Inflammatory Drugs: Studies in HTC Cultures. At least three systems of transport could be readily identified in HTC cultures. These included the "L" system, which was not Na^{+}- and energy-dependent and had a high affinity for BCH (K_m, 0.1 mM), and two Na^{+}-dependent systems which had an affinity for both AIB and MeAIB ("A" system). The two Na^{+}-dependent components could be readily distinguished by a marked difference in affinity constants for MeAIB (0.27 and > 3 mM, respectively) (Table I). Indomethacin inhibited selectively the high affinity component in a dose-dependent manner (between 0.05 and 0.4 mM). The Na^+-independent uptake of AIB and MeAIB was unaffected, and the uptake of BCH ("L" system) was enhanced by indomethacin (Table II).

All of the anti-inflammatory drugs tested inhibited the uptake of AIB and culture growth to a similar degree (Table III) and stimulated the uptake of BCH. Inactive derivatives or analogues of the drugs, such as gentistic acid and o-hydroxybenzoic acid, were neither cytostatic nor inhibitory (of amino acid transport).

Studies in other Cell Lines. The high affinity component of the "A" system was also identified in a BRL-T hepatocyte cell line (cloned from spontaneously transformed liver cells isolated from a Buffalo rat) and a nontransformed human fibroblast cell line. This component was especially prominent in the HTC and exponentially growing fibroblast cultures. It disappeared in confluent fibroblast cultures but decayed to some extent in confluent cultures of the two transformed cell lines. As with the HTC cultures, only the high affinity component was inhibited by indomethacin and uptake of BCH by the "L" system was enhanced (Table II).

Time Course of Inhibition of the High Affinity "A" Component: Studies with Cycloheximide. Upon addition of indomethacin, the uptake of MeAIB declined in an exponential fashion over the course of 2-4 hr. The time course was similar to that observed with cycloheximide, although, unlike cycloheximide, indomethacin's primary action did not appear to be through inhibition of protein synthesis.

Removal of drug led to the gradual reappearance of the high affinity component, and the uptake of MeAIB was fully restored (6-8 hr) before cells entered the "S" phase of their cycle as indicated by resumption of DNA synthesis.

Kinetic Analysis of the Effects of Indomethacin on Amino Acid Transport and Sodium Dependency. In all cell lines, indomethacin did not alter the affinity of the amino acids (AIB, MeAIB and BCH) for the transport system nor, in the case of MeAIB, the affinity of the carrier for Na^+. The only effect observed was a decline (or an increase in the case of BCH) in the values for V_{max}. We conclude from these data and the findings described in the previous section that indomethacin altered the number of functional transport carriers and that indomethacin's action was related in some manner to the normal turnover of carriers in the plasma membrane.

Uptake of Indomethacin by Cells and Influence of Binding to Serum in the Culture Medium. As reported in last year's report, studies with ^{14}C-indomethacin indicated that the drug was localized to a high degree intracellularly and that the localization was influenced by pH and serum concentration of the medium. Scatchard analysis revealed that indomethacin is bound by two processes, one with a high affinity for the drug and saturated at low drug concentration (50 μm), and one which was nonsaturable and had a low affinity for the drug. The latter was thought to be due to uptake of drug into lipid structures. In BRL-T cultures, which can be grown in low concentrations of serum, indomethacin's potency was found to be directly related with the concentration of serum. In the presence of 1% fetal calf serum, a 50% inhibition of growth was observed at 50 μM, a concentration that is close to that observed therapeutically (20 μM) or after pharmacological doses of drug in dog and rat (40 μM).

Amino Acid Transport in Thymus Lymphocytes Removed from Normal and Indomethacin-Treated Rats. Thymus lymphocytes from untreated rats took up amino acids in the same manner as the cell cultures, i.e., uptake by an "L" system and two Na^+-dependent components of the "A" system could be identified by differences in affinity constants for MeAIB. In cells isolated from indomethacin-treated rats (10 mg/kg, p.o., daily for 3 days), the high affinity component of the "A" system was largely suppressed. Addition of indomethacin to lymphocytes from control rats resulted in a decay in uptake of MeAIB. Enhancement of the high affinity component (by incubation in amino acid-free medium) was suppressed by indomethacin.

Significance to Biomedical Research. The work reveals a valuable property of the anti-inflammatory drugs; that is, they arrest cell growth in the G_1 phase for up to several days and that after removal of drug synchronous growth of culture occurs. The ability to suppress the emergence of the high affinity of amino acid transport may be related to the ability of these drugs to inhibit cell cycling and lymphocyte transformation. These effects may have therapeutic significance and may have potential use in studies of cell cycle.

Proposed Course: Reactions associated with changes in membrane function, e.g., methylation of phospholipids, energy coupling, will be investigated as possible mechanisms for the decay in active transport.

Publications:

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Bayer, B.M., Lo, T.N., and Beaven, M.A.: Anti-inflammatory drugs alter amino acid transport in HTC cells. J. Biol. Chem., in press.

Beaven, M.A. and Bayer, B.M.: Factors influencing the uptake and disposition of indomethacin-^{14}C in cell cultures. Biochem. Pharmacol., in press.

TABLE I

Kinetic parameters of AIB, MeAIB, and BCH transport in the presence of indomethacin

	Inhibitable component		Non-Inhibitable Component	
	K_m	V_{max}	K_m	V_{max}
AIB	0.7	4.5 (7.5)	--	--
MeAIB	0.27	2.7 (5.5)	--	--
BCH	--	--	0.11	3.1 (1.95)

K_m = mM; V_{max} = nmole/10^6 cells/10 min.

TABLE II

Influence of indomethacin on uptake of amino acids in the presence and absence of Na^+

Medium	Treatment	Uptake (pmoles/10^6 cells/10 min)		
		AIB	CH_3-AIB	BCH
Complete Krebs Ringer	Control	2.57 ± 0.13	149.2 ± 6.6	1036 ± 21.6
	Indomethacin (0.4 mM)	1.00 ± 0.03 (-61%)	55.8 ± 2.2 (-63%)	1355 ± 6.6 (+30%)
Na^+-Free Krebs Ringer	Control	0.21 ± 0.002	3.0 ± 0.2	1065 ± 23.0
	Indomethacin (0.4 mM)	0.20 ± 0.003 (-7%)	2.4 ± 0.2 (-18%)	1253 ± 20.8 (+18%)

Mean ± SEM (n = 6).

TABLE III

Effect of different anti-inflammatory drugs on amino acid uptake

Drug	(mM)	Uptake	
		$AIB-^3H$	$BCH-^{14}C$
		(% of controls)	
Indomethacin	0.2	71 ± 4	94 ± 2
	0.4	48 ± 3	105 ± 2
Sodium meclofenamate	0.5	53 ± 3	102 ± 4
Phenylbutazone	1.0	44 ± 2	116 ± 1
	0.5	97 ± 8	100 ± 2
Aspirin	1.0	112 ± 4	94 ± 1
	7.5	73 ± 8	97 ± 3
	10.0	65 ± 4	95 ± 4
Gentisic acid	5.0	112 ± 8	106 ± 3
	10.0	123 ± 12	114 ± 2
	15.0	147 ± 8	115 ± 3

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00622-03 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Cyclic Nucleotide Metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI
OTHER:	Peter H. Fishman	Biochemist	DMN	NINCDS

COOPERATING UNITS (if any)
Dr. M. D. Lane, Department of Physiological Chemistry, Johns Hopkins University School of Medicine, Baltimore, Md.

LAB/BRANCH
Cellular Metabolism

SECTION
Molecular Mechanisms

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.4	0.8	1.6

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) The ability of choleragen preparations to catalyze the activation of adenylate cyclase, the ADP-ribosylation of arginine and protein, and the hydrolysis of NAD is an intrinsic activity of the A_1 peptide. Choleragen is thus capable of activating the ribosyl-nicotinamide of NAD in the absence of the physiological acceptor and is similar to E. coli heat-labile enterotoxin, Pseudomonas Exotoxin A, and diphtheria toxin. 2) Differentiation of the 3T3-L1 preadipocyte is associated with loss in ganglioside content, a decrease in the choleragen receptor, ganglioside G_{M1}, and a reduction in surface G_{M1} as estimated by choleragen binding or fluorescent staining of bound choleragen. G_{M1} thus serves as a marker for the transition from fibroblast to adipocytes.
3) An ADP-ribosyltransferase, which catalyzes the stereospecific ADP-ribosylation of arginine and protein and, to a lesser extent, the hydrolysis of NAD, was identified in avian erythrocytes and purified > 500,000-fold with an 18% yield. One protein band of 28,300 molecular weight was observed on sodium dodecyl sulfate-polyacrylamide gels. Activity cochromatographed with protein on gel permeation columns. The turnover number of the purified protein was 9,000 moles min^{-1} mole enzyme^{-1}.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To define the factors that regulate cyclic nucleotide metabolism in animal cells. Cyclic nucleotide synthesis and degradation are regulated in part by mammalian hormones and their analogs and bacterial toxins. Activation of adenylate cyclase by these agents involves their interaction with a specific surface receptor and subsequently, in the case of two toxins, the enzymatic modification of a cellular protein. The enzymatic mechanism was studied for the bacterial toxins, and a similar process was defined in erythrocytes.

Methods Employed: NAD glycohydrolase and ADP-ribosyltransferase activities were determined by assays developed in the laboratory.

Major Findings: 1) Activation of Adenylate Cyclase by ADP-Ribosylation Catalyzed by Choleragen. Choleragen appears to exert its effects on cells through the NAD-dependent activation of adenylate cyclase. A possible role for NAD as a substrate in an ADP-ribosylation reaction was suggested by our initial observation that choleragen catalyzes NAD hydrolysis as well as the ADP-ribosylation of arginine and proteins. In a recent report, Tait and van Heyningen stated that the NAD glycohydrolase activity found in choleragen preparations is due to a contaminating enzyme. The ability of choleragen to catalyze NAD hydrolysis, however, is of importance to an understanding of the mechanism of toxin action. The presence of NAD glycohydrolase activity demonstrates that the toxin can activate the ribosylnicotinamide bond of NAD in the absence of an acceptor protein and can use water as an ADP-ribose acceptor.

Since work on two other NAD-dependent bacterial toxins, diphtheria toxin and Pseudomonas Exotoxin A, is consistent with the hypothesis that NAD hydrolysis occurs in the absence of a protein acceptor and since our previous findings were consistent with the A subunit of choleragen possessing an intrinsic NAD glycohydrolase activity, we investigated the ability of purified peptides from choleragen to activate adenylate cyclase and catalyze NAD hydrolysis.

Choleragen consists of three peptides: A_1, A_2, and B. The A_1 peptide is joined to A_2 through a single disulfide bond. The two peptides can be separated by gel permeation chromatography after alkylation of the -SH groups or by polyacrylamide gel electrophoresis in Triton X-100 and dithiothreitol.

In agreement with our prior observations, the purified A_1 peptide of choleragen, but not the A_2 or B, catalyzed the hydrolysis of NAD to ADP-ribose and nicotinamide and the NAD-dependent ADP-ribosylation of arginine. Both alkylated and unmodified A_1 catalyzed the activation of adenylate cyclase and possessed NAD glycohydrolase and ADP-ribosyltransferase activity. All the NAD glycohydrolase activity of this choleragen preparation co-electrophoresed with toxin protein and cochromatographed with the A_1 peptide, the ADP-ribosyltransferase activity, and the ability to activate rat liver adenylate cyclase. The K_m for NAD was similar for the A_1 peptide to that of the intact toxin which contains one A_1 per molecule. In contrast to the holotoxin or its A subunit, the A_1 peptide did not exhibit a lag in reaction rate or a thiol requirement for activity.

Since alkylated A_1 is as active as holotoxin, it would appear that the cysteine is not critical for enzymatic activity. Ledley et al. have noted a sequence homology between the A_1 of choleragen and the α subunit of the glycopeptide hormones which involved the cysteine and two adjacent amino acid residues and suggested that this common sequence might imply a similar mechanism of action of both toxin and hormone. Since an intact cysteine is not required for enzymatic activity, however, it is unlikely that the sequence homology between A_1 and α subunit occurs in a critical region and is related to an ability to catalyze similar enzymatic reactions.

These results support the conclusion that the A_1 peptide of choleragen possesses NAD glycohydrolase activity and can activate the ribose-nicotinamide bond of NAD in the absence of an acceptor protein.

2) Loss of Choleragen Receptors and Ganglioside upon Differentiation of 3T3-L1 Preadipocytes. 3T3-L1 cells, cloned by Green and Kehinde from mouse embryo fibroblasts, differentiate in culture into cells exhibiting typical adipocyte morphology. The differentiation of 3T3-L1 preadipocytes is accompanied by changes consistent with the increased lipogenic capabilities of the cell.

As 3T3-L1 preadipocytes arose from a 3T3-fibroblast line, their differentiation appears to represent a transition from fibroblast to adipocyte. Thus, the loss of certain membrane components during 3T3-L1 differentiation is anticipated based on reported differences in the membrane composition of fibroblasts and adipocytes. Ganglioside G_{M1} is present in 3T3 fibroblasts but can be detected only with special methodology at low levels in rat adipocytes as well as adipose tissue from other species, including mouse. Changes in G_{M1} content may thus provide a useful marker for differentiation from fibroblast to adipocyte.

During the differentiation of the 3T3-L1 fibroblast, changes were observed in ganglioside content. Differentiation was accompanied by a decrease in total cellular ganglioside content; the ganglioside level was 1.8- to 2.5-fold higher in undifferentiated than in differentiated cells. Gangliosides G_{M3} and G_{D1a} constituted a majority of total cell gangliosides in both cell types, while ganglioside G_{M1}, the putative choleragen receptor, constituted $< 5\%$. Differentiation resulted in a 75 to 85% decrease in ganglioside G_{M1}.

An inverse correlation existed between the percentage of adipocytes in the cell populations and: 1) total ganglioside and ganglioside G_{M1} content and 2) surface ganglioside G_{M1} as estimated by choleragen binding or fluorescent staining of bound choleragen. Nondifferentiating 3T3-C2 control cells did not exhibit changes in total ganglioside, ganglioside G_{M1}, or choleragen binding that were observed with 3T3-L1 cells.

3) Isolation and Properties of an NAD-and Guanidine-Dependent ADP-Ribosyltransferase from Turkey Erythrocytes. The activation of adenylate cyclase by two bacterial toxins from Vibrio cholerae and Escherichia coli has been shown to involve the NAD-dependent ADP-ribosylation of a cellular protein by a subunit of the toxin. The relationship of this cellular protein to the catalytic unit of the cyclase remains to be clarified. These two toxins catalyze, in addition to the ADP-ribosylation of cellular proteins, the hydrolysis of NAD and the NAD-dependent ADP-ribosylation of arginine and other guanidine derivatives.

An NAD- and guanidine-dependent ADP-ribosyltransferase was purified more than 500,000-fold from turkey erythrocytes with an 18% yield. The enzyme in the 100,000 x g supernatant fraction was bound to phenyl-Sepharose, eluted with 50% propylene glycol, and further purified by sequential chromatographic steps on carboxymethyl cellulose, NAD-Agarose and Concanavalin A-Agarose. The transferase was specifically eluted from Concanavalin A-Agarose with α-methylmannoside. This procedure resolved the guanidine-dependent ADP-ribosyltransferase from the guanidine-independent NAD glycohydrolases. The enzyme was highly unstable, both in routine storage and in the assay, but could be stabilized by the addition of 30 to 50% glycerol or propylene glycol to the storage buffer and by the addition of nontransferase protein to the assay. Significant increases in enzyme recovery were obtained by conducting the NAD- and Concanavalin A-Agarose chromatography in buffer containing propylene glycol. Glycerol or propylene glycol in the assay was inhibitory.

The enzyme used both NAD and NADP as ribose donors; K_m values for the two were similar (30 μM), although the V_{max} with NAD was considerably higher than that with NADP. The K_m for NAD, 30 μM, is significantly lower than the K_m values of 4 mM and 8 mM noted with choleragen and E. coli enterotoxin, respectively. With choleragen, NADP functioned poorly in the activation of the pigeon erythrocyte and rat liver adenylate cyclases and was hydrolyzed at less than 1% the rate observed with NAD in the NAD glycohydrolase and ADP-ribosyltransferase assays. Thus, the catalytic site of choleragen discriminated between NAD and NADP more stringently than did the avian erythrocyte transferase. This ability of the avian enzyme to utilize NADP as an ADP-ribose donor, though different from choleragen, is similar to that of some mammalian NAD glycohydrolases.

The K_m values for arginine methyl ester were 2 and 50 mM, while those for arginine were 6 and 75 mM; the Lineweaver-Burk plots were consistent with "negative cooperativity." In addition to arginine, other guanidino derivatives served as ADP-ribose acceptors with agmatine = arginine methyl ester > arginine > guanidine > guanidinobutyrate > guanidinopropionate. The stereospecificity of the transferase-catalyzed reaction between NAD and arginine was established by NMR spectroscopy; the α-anomeric species of ADP-ribose-L-arginine ester was formed preferentially. Similar stereospecificity was observed with both choleragen and E. coli heat-labile enterotoxin.

The purified protein exhibits one predominant protein band on SDS-polyacrylamide gels with an estimated molecular weight of 28,300. On Ultrogel

AcA 54 chromatography, single coincident peaks of ADP-ribosyltransferase activity and protein were observed. Enzyme activity was independent of DNA. The specific activity of the purified enzyme (350 μmol of ADP-ribose transferred from NAD to argine methyl ester$\cdot$min$^{-1}\cdot$mg^{-1}) (using arginine methyl ester as the ADP-ribose acceptor) is considerably higher than that obtained with either toxin. Since arginine methyl ester is presumably only a model for the ADP-ribose acceptor(s) in cells, the maximal velocity for either the transferase or choleragen or both may be higher with the natural intracellular acceptor(s).

The specific activity and estimated protomeric molecular weight translated into a turnover number of ∿ 9,000 mol of ADP-ribose transferred to arginine methyl ester per min per mol enzyme. This activity is comparable to that of NAD glycohydrolases and is higher than that reported for poly(ADP-ribose)transferases. The latter enzymes can be presumed to catalyze multiple distinct reactions, and the transfer of ADP-ribose to protein may not represent the rate-limiting step.

The highly purified transferase was inhibited by thymidine, nicotinamide and theophylline. The erythrocyte (ADP-ribosyl) transferase was thus similar to the poly(ADP-ribose)polymerase in its sensitivity to inhibition by nicotinamide and thymidine. The fact that these agents inhibit ADP-ribosylation is consistent with the possibility that some of their effects in cells may result from inhibition of mono- as well as poly(ADP-ribosylation).

Although the function of ADP-ribosylation in the action of bacterial toxins has been partially defined, the role of endogenous NAD-dependent mono(ADP-ribosylation) in animal cells remains unclear. The availability of a purified ADP-ribosyltransferase should assist in the investigation into this form of covalent modification.

Significance to Biomedical Research: The pulmonary and cardiovascular systems are affected under physiological and pathological conditions by extracellular agents such as hormones and toxins. The lung, in particular, is exposed through the tracheobronchial tree to a variety of bacterial and toxic agents. To be effective, some of these agents must interact with a cellular receptor, and a substantial number of these agents exert their effects by altering the steady-state levels and localizations of cyclic nucleotides within the cell. A number of pharmacological agents have been in use which override aberrant physiological control to the benefit of the patient; these agents have been designed to interact with specific cellular receptors. By using cultured cells, model systems and purified preparations, it may be possible to simplify and define the factors critical to cyclic nucleotide metabolism. These models can then be used to understand the controls which operate in the more complex pulmonary and cardiovascular system.

Proposed Course: The events which occur following the binding of hormone on toxin and the role of covalent modification in the action of hormone on toxin will be defined.

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Vaughan, M. and Moss, J.: ADP-ribosylation and Activation of Adenylate Cyclase. In Proceedings of the Fourth US-USSR Symposium on Myocardial Metabolism, September 14-16, 1979, Tashkent, USSR, in press.

Moss, J. and Vaughan, M.: Specificity of ADP-ribosylation by Choleragen (Cholera Toxin), *E. coli* Heat Labile Enterotoxin and an Arginine- and NAD-dependent Avian Erythrocyte Transferase. In Proceedings of the Conference on Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Bethesda, Maryland, October 22-24, 1979, in press.

Fishman, P.H., Pacusza, T., Hom, B.E., and Moss, J.: Modification of ganglioside G_{M1}: Effect of lipid moiety on choleragen action. J. Biol. Chem., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00623-02 CM

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mevalonate Pyrophosphate Decarboxylase in Human Skin Fibroblasts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Avigan	Research Chemist	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cellular Metabolism

SECTION

Metabolic Regulation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.2	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A method for assay of the activity of mevalonate pyrophosphate decarboxylase in fibroblasts was developed. In similarity to earlier findings with mevalonate kinase, the activity of mevalonate pyrophosphate decarboxylase was increased when whole serum was replaced with lipid-deficient serum in the incubation medium. Unlike mevalonate kinase, mevalonate pyrophosphate decarboxylase activity was not increased by insulin.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: This project is a continuation of and it completes the previous year's project No. Z01 HL 00623-01 CM. Its purpose was to explore the potential role of enzymes other than Hydroxymethylglutaryl Coenzyme A reductase in the regulation of sterol synthesis in mammalian tissues. The decarboxylase step follows, in the metabolic sequence, mevalonate phosphorylation, the study of which was reported earlier.

Methods Employed: Human skin fibroblasts are grown in monolayers. Cells are incubated in a medium containing various additions, washed, harvested and frozen. Cells are homogenized, and the 10,000 x g supernatant of the homogenate is used for the mevalonate pyrophosphate assay. The radioactive substrate is prepared by incubation of 2-^{14}C-mevalonate with pig liver enzymes and isolated by TLC.

Major Findings: Labeled mevalonate pyrophosphate was readily converted to isopentenyl pyrophospahte under the conditions of the assay (enzymatic decarboxylation). Mevalonate pyrophosphate decarboxylase, like mevalonate kinase, was 34% lower in cells incubated with whole serum than in those incubated with lipid-deficient serum. Insulin, which increases mevalonate kinase activity ∿ 100%, had no significant effect on mevalonate pyrophosphate decarboxylase in these cells.

Significance to Biomedical Research: The inhibitory effect of serum lipids on mevalonate pyrophosphate decarboxylase is likely to contribute to feedback inhibition of overall cholesterol synthesis in the organism.

Proposed Course: This project is esssentially completed and the results are being prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00625-02 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

ADP-Ribosyltransferases: Characterization of their Substrates and of Factors that Control their Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Paul A. Watkins	Research Associate	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of GTP in choleragen-dependent activation of adenylate cyclase was studied by examining the effect of nucleoside triphosphates on choleragen-catalyzed ADP-ribosylation of proteins. Choleragen catalyzed the transfer of ADP-ribose from NAD to multiple soluble and membrane proteins of bovine thymus and brain as well as to purified proteins such as polyarginine, lysozyme, and histone. Nucleoside triphosphates, primarily GTP, enhanced the labeling of several soluble proteins, and had variable effects on ADP-ribosylation of purified proteins.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Cholera enterotoxin and an enzyme purified from turkey erythrocytes catalyze the NAD-dependent ADP-ribosylation of proteins. The purpose of this study was to 1) identify specific cellular proteins that serve as substrates for choleragen and transferase, 2) examine the effect of ADP-ribosylation of cellular proteins on metabolic processes, and 3) examine mechanisms for the regulation of toxin- and transferase-catalyzed ADP-ribosylation of proteins.

Methods Employed: Purified proteins or fractions from bovine thymus or brain were incubated with ^{32}P- or ^{3}H-NAD; proteins were precipitated with acid, collected on filters, solubilized, and separated on polyacrylamide slab gels containing SDS. ADP-ribosylated proteins were digested with snake venom phosphodiesterase to study the nature of the covalent modification.

Major Findings: Choleragen exerts its toxic effects on animal cells through activation of adenylate cyclase with a resulting increase in intracellular cAMP content. In cell-free systems, activation of adenylate cyclase requires NAD and GTP. NAD serves as an ADP-ribose donor for the choleragen-catalyzed ADP-ribosylation of membrane components and several purified proteins. GTP is necessary for optimal catalytic activity of hormone- and toxin-activated adenylate cyclases, leading to the suggestion that a GTP-binding protein is a regulatory component of the cyclase system. A guanylnucleotide-binding protein of molecular weight ~ 42,000 has been partially purified in other laboratories from pigeon erythrocyte membranes. It conferred GTP sensitivity on adenylate cyclase preparations that were otherwise unresponsive to nucleotide. Other investigators have shown that several pigeon erythrocyte and mouse lymphoma membrane proteins can be ADP-ribosylated by choleragen in the presence of cytosolic factors and nucleoside triphosphate; the principal one has a molecular weight similar to that of the GTP-binding protein. In all systems, choleragen apparently catalyzed the nucleoside triphosphate-dependent ADP-ribosylation of multiple proteins. We questioned, therefore, whether the GTP dependence of the reaction was of itself sufficient to implicate an ADP-ribosylated protein in adenylate cyclase regulation.

Choleragen, using [^{3}H] or [^{32}P]NAD as substrate, catalyzed the ADP-ribosylation of soluble and membrane proteins of bovine thymus and brain. Choleragen-dependent ADP-ribosylation of soluble proteins from bovine thymus, using [^{32}P]NAD as substrate, was increased three- to fourfold by GTP. The effect was specific for nucleoside triphosphate, with GTP $\cong$ ITP > CTP > ATP > UTP. Half-maximal enhancement was observed with 0.5 mM GTP. The magnitude of the GTP effect decreased with increasing NAD concentration; GTP had no effect on hydrolysis of NAD at low NAD concentrations. Digestion of ADP-ribosylated proteins with snake venom phosphodiesterase yielded primarily 5'-AMP, indicating that the product is a mono- rather than a poly(ADP-ribosyl)-protein. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of soluble proteins from thymus after incubation with choleragen and [^{32}P]NAD separated numerous ADP-ribosylated proteins; radioactivity in all bands was increased by nucleoside triphosphate. Choleragen catalyzed the ADP-ribosylation of several purified proteins; depending on the protein, GTP either increased, decreased, or had no effect on the extent of ADP-ribosylation. GTP virtually abolished labeling of polyarginine, nearly doubled the amount of label incorporated into lysozyme and calf thymus histone classes, f_{2a}, f_{2b}, and f_3, and had no effect when several other proteins served as ADP-ribose acceptors. The fact that choleragen catalyzes the ADP-ribosylation of a wide variety of proteins is consistent with the possibility that intoxication results in the covalent modification of more than one cellular protein and perhaps alters the activity of enzymes in addition to adenylate cyclase.

Significance to Biomedical Research: Choleragen exerts its effects on mammalian cells by activating adenylate cyclase, thereby increasing intracellular cAMP levels. This presumably occurs via the toxin-catalyzed ADP-ribosylation of a component of the adenylate cyclase system. The elucidation of the nature of the specific cyclase component modified by choleragen will greatly extend present knowledge of the regulation of cyclic nucleotide metabolism. Moreover, the presence of an endogenous enzyme in turkey erythrocytes that catalyzes a reaction identical to that catalyzed by cholera toxin suggests that a similar mode of regulation may exist endogenously. Since cyclic nucleotides are known to regulate numerous metabolic processes in tissue such as heart and lung, knowledge of the regulation of cAMP levels would be valuable. In addition, the fact that several proteins are ADP-ribosylated by choleragen and that this process may be modulated *in vivo* by nucleoside triphosphates suggests that ADP-ribosylation of proteins may be a generalized mechanism of metabolic regulation.

Proposed Course: 1) Identification of the protein substrates for choleragen-catalyzed ADP-ribosylation in intact cells.

2) Determination of the effects of nucleoside triphosphates on ADP-ribosylation catalyzed by the turkey erythrocyte transferase-catalyzed reaction.

Publications: Watkins, P.A., Moss, J., and Vaughan, M.: Effects of GTP on choleragen-catalyzed ADP-ribosylation of membrane and soluble proteins. J. Biol. Chem. 255: 3959-3963, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00627-02 CM

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of GTP-Binding Protein in the Regulation of Adenylate Cyclase Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan J. Bitonti	Staff Fellow	CM	NHLBI
	Seishi Nakaya	Visiting Associate	CM	NHLBI
	Joel Moss	Head, Section on Molecular Mechanisms	CM	NHLBI
	Martha Vaughan	Chief, Laboratory of Cellular Metabolism	CM	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Cellular Metabolism

SECTION

Molecular Mechanisms

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) Many cells contain a GTP-binding protein which is part of the adenylate cyclase complex. The GTP-binding protein is involved in the regulation of adenylate cyclase by hormones, cholera toxin, and other agents. We are attempting to isolate the nucleotide binding protein from porcine erythrocyte membranes. We are using a variant of S49 mouse lymphoma cells which are deficient in GTP-binding protein to assess the progress of the purification.

2) Complementation of the S49 cell membranes deficient in GTP-binding protein with the factor was time- and temperature-dependent and enhanced by prior activation of the factor with choleragen and GTP or a non-hydrolyzable GTP analogue.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Numerous cells contain a GTP-binding protein which is believed to be involved in the regulation of adenylate cyclase by hormones, catecholamine and cholera toxin. As a prerequisite to establishing the role of the protein to this regulation, we are attempting to purify the GTP-binding protein from porcine erythrocyte membranes and to clarify the factors involved in the interaction of this protein with the other components of the cyclase system.

Methods Employed: Porcine erythrocyte membranes are prepared by hemolysis and extensive washing in hypotonic buffers at neutral pH. The purified membranes are solubilized with detergents followed by centrifugation at 100,000 x g for 60 min. The GTP-binding protein is determined by reconstitution with variant S49 lymphoma cell membranes which are genetically deficient in GTP-binding protein (Ross et al., J. Biol. Chem. 252: 5761, 1977). The lymphoma cell membranes have no demonstrable adenylate cyclase activity unless preincubated with detergent extracts of membranes from various cells. Adenylate cyclase was assayed by the procedure of Salomon and Rodbell (Anal. Biochem. 58: 541, 1974).

Major Findings: 1) The GTP-binding protein has been partially purified by using several chromatographic techniques. Those which have proven most useful are ion-exchange chromatography on DEAE, hydrophobic chromatography on octyl Sepharose, and gel filtration using Ultrogel AcA34. The protein has an apparent molecular weight of 130,000 as determined by gel filtration, although this is dependent on detergent concentration. SDS-polyacrylamide gel electrophoresis of detergent extracts of porcine erythrocyte membranes pretreated with cholera toxin and ^{32}P-NAD reveals two major proteins which are labeled with ^{32}P. One protein has a molecular weight of 50-55,000 and the other has a molecular weight of 42,000.

2) Complementation of the GTP-binding protein with the lymphoma cell membranes was time-dependent and favored by increasing temperature from 0°C to 37°C. The GTP-binding protein needed to be activated by either choleragen, GTP and NAD or by Gpp(NH)p. The solubilized GTP-binding protein appeared to recombine with membranes from the lymphoma cells better than did the GTP-binding protein when still present in an intact membrane.

Significance to Biomedical Research: Many hormones, drugs, and other agents exert their effects on cells by modifying adenylate cyclase activity, but the molecular mechanisms by which this important enzyme is regulated remain to be elucidated.

Proposed Course: To define the relationship of the GTP-binding protein to adenylate cyclase and its role in the regulation of cyclase activity.

Publications: Bitonti, A.J., Moss, J., Tandon, N.N., and Vaughan, M.: Prostaglandins increase GTP hydrolysis by membranes from human mononuclear cells. J. Biol. Chem. 255: 2026-2029, 1980.

Nakaya, S., Moss, J., and Vaughan, M.: Analysis of cofactor requirements for activation of adenylate cyclase by choleragen independent of requirements for catalytic activity. In Proceedings of the Fifteenth Joint Conference on Cholera. DHEW Publ. No. (NIH) 80-2003, 1980, pp. 299-306.

Nakaya, S., Moss, J., and Vaughan, M.: Effects of nucleoside triphosphates on choleragen-activated brain adenylate cyclase. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00629-01 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Presence of Histamine in Brain Neurones: A Phylogenetic Study

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alvair P. Almeida	Guest Worker	CM	NHLBI
	Michael A. Beaven	Head, Section on Cellular Pharmacology	CM	NHLBI

COOPERATING UNITS (if any)
Dr. Almeida is an International Research Fellow, Fogarty International Center, NIH. Dr. John Valois, Woods Hole Marine Laboratories, Woods Hole, Mass.

LAB/BRANCH
Cellular Metabolism

SECTION
Cellular Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)
Although histamine is localized in regions that represent phylogenetically older parts of mammalian brain, there has been no systematic study of its occurrence in brain of primitive vertebrates. Histamine was found in brain of all twelve vertebrate species tested. Species which did not have a cerebral cortex (neopallium) or a well-developed cerebellum, such as amphibia, fish and hagfish, had higher cerebral histamine levels than mammalian species, even though histamine was present in only trace (< 0.1 μg/g tissue) amounts in most peripheral tissues (an exception was gastric mucosa) of these species. In dogfish, histamine was present throughout the brain stem and spinal cord but was mostly concentrated in the midbrain region. A large proportion of this histamine could be recovered in washed synaptosomal preparations. Histamine methyltransferase and diamine oxidase activity was present in brain of all aquatic species, whereas only methyltransferase was present in mammalian species. The studies add further support to the idea that histamine is a neurotransmitter and suggest that histaminergic neurones innervate primitive regions of the brain.

Project No. Z01 HL 00629-01 CM

Project Description:

Objectives: In addition to histamine's participation in anaphylactic reactions and in processes involved in gastric secretion, there has been increasing interest in its possible role in brain. Studies since 1966 have shown that histamine is highly localized in certain parts of mammalian brain, particularly in discrete nuclei in the hypothalamus. Much of this histamine can be recovered in synaptosomal fractions of brain homogenates. More recently, histamine receptors (both H_1 and H_2) have been identified in rat and guinea pig brain by the binding of labeled agonists and antagonists to synaptosomal membranes and by histamine-induced activation of adenylate cyclase in brain slices. Histamine, when administred into brain by iontophoretic techniques, causes either suppression (through activation of H_2 receptors) or facilitation (through activation of H_1 receptors) of spontaneous neuronal firing in the hypothalamus and various other structures in the diencephalon and forebrain. Somatic responses include a decrease in blood pressure (up to 50 mm Hg), an increase in heart rate (up by 30%) and a marked decrease in body temperature. From measurements of histamine and histidine decarboxylase activity in microbiopsy specimens, Schwartz and his collaborators (see Life Sci. 25: 895-912, 1979) have concluded that medial bundles of histaminergic fibers pass through the midbrain and innervate synapses in the forebrain. However, release of endogenous histamine and associated changes in neurophysiological parameters has not been demonstrated.

The above studies have utilized mammalian brain, and little data are available for nonmammalian species. Since histamine is located almost exclusively in the phylogenetically older parts of mammalian brain, we felt that if histamine has a role in neurotransmission it should be present in brain of more primitive veretebrate species. This program was undertaken to verify this thesis.

Methods Employed: Sources. Mammalian species were obtained through NIH sources, and amphibia were borrowed from other investigators in the Institute. Fish heads were cut from freshly caught specimens by Dr. John Valois at the Woods Hole Marine Laboratories, and these were shipped to us on Dry Ice. Brains were dissected directly from the frozen specimens. Hagfish and amphioxus were purchased live from Pacific Bio-Marine Lab., Inc., Venice, CA. These species were frozen on Dry Ice before dissection. Brain, skin, muscle and a proximal section of the intestine were removed from all species.

Methods. Histamine, histamine methyltransferase, and histidine decarboxylase activity were measured by isotopic microassays as described in previous publications (see Beaven, M.A., Wilcox, G., and Terpstra, G.K., Anal. Biochem. 84: 638-641, 1978; Shaff, R.E. and Beaven, M.A., Anal. Biochem. 94: 425-430, 1979).

Major Findings: Histamine Content of Brain and Peripheral Tissues of Various Chordates. The brain of all species examined contained histamine.

In mammalian species, the histamine content varied from 80-130 ng/g of whole brain. In lower species, the histamine content varied from 230 ng/g to 750 ng/g (see Table) in amphibia and fish and 108 ng/g in hagfish brain. Cerebral spinal fluid, where this was measured, was less than 10 ng/g fluid. In contrast, the terminal end of the spinal cord of the hemichordate, amphioxus, contained less than 25 ng histamine/g tissue.

As reported by others, peripheral tissues of the aquatic species contained little histamine (< 700 ng/g), whereas skin, intestine and muscle in the mammalian species contained high but variable (between 1,000 and 30,000 ng/g tissue) histamine levels (see Table).

Distribution of Histamine-Metabolizing Enzyme in Vertebrate Brain. Histidine decarboxylase activity, although low in whole rat brain, was present in significant amounts in the hypothalamus. The activity in dogfish brain, however, was too low to permit adequate identification.

Of the two enzymes responsible for histamine inactivation, histamine methyltransferase was found in high activity in brain of all species. Diamine oxidase was absent in brain of mammals but was present to a variable extent in brain of the amphibian and fish species. None of these enzyme activities could be detected in the spinal cord of amphioxus.

Distribution of Histamine in Dogfish Brain. Gross dissection of two dogfish brains showed that the highest histamine levels were in the midbrain (diencephalon) and were highest in the hypothalamus (~ 300 ng/g tissue). Cerebellum, medulla and the rhombocephalon contained 135, 195 and 179 ng/g tissue, respectively, and the cerebrum (paleopallium) 90 ng/g tissue. Appreciable quantities (153 ng/g) were also present in spinal cord but only trace amounts (3 ng/g) in the cranial fluids.

Washed synaptosomes prepared from the midbrain contained 60 ng histamine/g of original brain or about 33% of the original histamine content. Preliminary studies with highly labeled ^{3}H-mepyramine indicate selective binding of the ligand to midbrain-neuronal membranes, a possible indication of H_1 receptors in this region.

Conclusions and Significance to Biomedical Research: The phylogenetic studies of Reite (Physiol. Rev., 1972) showed that histamine is localized in stomach of all species with a well-defined stomach (i.e., cartilaginous fishes and higher vertebrates) and in mast cells in animals with a terrestrial habitat (i.e., reptiles, birds and mammals). The studies reported here are the first evidence of histamine's early appearance in brain during the evolution of vertebrate species. The low histamine content of amphioxus spinal cord, the higher content in hagfish brain and spinal cord, and the high degree of localization of histamine in midbrain structures of dogfish suggest that histamine is associated with the development of the brain stem. The relatively high levels of histamine in whole brain of nonmammalian species compared to those in brain of mammalian species, in which the cerebral cortex (neopallium) and cerebellum account for most of the brain mass, also suggest that the histaminergic neurones remain restricted to the more primitive part of the limbic system. The recovery of histamine in synaptosomes of dogfish is consistent with the notion that histamine is located in neurones.

Other conclusions from this study are that 1) histamine methyltransferase has a major role in the inactivation of histamine in brain throughout evolution but that diamine oxidase may be an additional means of inactivation in lower species and 2) histamine levels in peripheral tissues (other than the G.I. tract) may be even lower than those reported by Reite (generally 100-2,000 ng/g), who used a less specific (fluorometric) method of assay.

Proposed Course: This will include future measurements of histamine in different brain regions and identification of the location of histamine receptors and the histamine-metabolizing enzymes (e.g., neurones, choroid plexus, blood vessel endothelial cells, etc.) in brain of different species.

Publications: None.

TABLE

Distribution of histamine in brain and other tissues in various chordate species

Species	(n)	Histamine (ng/g tissue)					
		Brain	Spinal cord	Cranial fluid	Skin	Intestine	Muscle
Mammals							
Rat (Sprague-Dawley)	(6)	80±5	---	---	26,200±6,000	11,000±2,000	4,700±1,500
Mouse (General purpose)	(6)	81±7	---	---	19,000±2,000	---	---
Guinea pig (Hartley)	(5)	130±7	---	---	4,000±500	13,000±1,000	3,900±900
Hamster (Syrian)	(5)	123±13	---	---	---	---	---
Amphibia							
Toad[1]	(5)	328±24	---	---	79±7	692±69	14±2
Mud puppy[2]	(5)	391±48	---	---	29±4	127±13	8±2
Bony fish							
Flounder[3]	(4)	236±46	---	9±1	33±5	---	13±5
Bluefish[4]	(5)	745±86	---	---	---	---	---
Sculpin[5]	(4)	312±59	247±32	4±1	25±5	---	41±17
Scup[6]	(5)	395±30	---	---	---	---	---
Cartilaginous fish							
Spiny dogfish[7]	(9)	302±30	108±14	3±1	36 (2)	---	78 (2)
Jawless fish							
Hagfish[8]	(5)	108±6	151	---	117±31	116±23	199±26
Hemichordate							
Amphioxus[9]	(2)	---	22	---		whole body 110	

[1] *Bufo marinus*

[2] *Necturus maculosus*

[3] *Pseudopleuronectes americanus*

[4] *Pomatomus saltatrix*

[5] *Myoxocephalus octodecemspinosus*

[6] *Stenostomus versicolor*

[7] *Squalus acanthius*

[8] *Myxine glutinosa*

[9] *Branchiostoma*

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00630-01 CM

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism of Lipids in Human Fibroblasts and Muscle Cells Grown in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Joel Avigan	Research Chemist	CM	NHLBI

COOPERATING UNITS (if any)
Medical Neurology Branch, NINCDS. Neonatal and Pediatric Medicine Branch, NICHHD.

LAB/BRANCH
Cellular Metabolism

SECTION
Metabolic Regulation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.7	0.8	0.9

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1) The effect of L-carnitine on the rate of oxidation of 1-^{14}C-palmitic acid by human skin fibroblasts and skeletal muscle cells grown in culture was studied. Incubation of cells for 24 hr with 0.5-10 mM L-carnitine increased the rate of substrate oxidation to CO_2 by up to 50% in normal fibroblasts and by as much as 100% in muscle cells. Fibroblasts from two patients with systemic carnitine deficiency and two patients with adrenomyeloneuropathy responded to L-carnitine in a manner similar to control fibroblasts. Uptake of ^{14}C-carnitine by skin fibroblasts from the two patients with carnitine deficiency was not lower than the uptake of control cells. 2) A study of metabolism of very long chain fatty acids in human cells in culture was initiated. 3) It was shown that dexamethasone consistently stimulates oxidation of labeled palmitic acid in fibroblasts. 4) Hybrid cells were produced by fusion of mouse myeloma and spleen lymphocytes from a mouse immunized against human skin fibroblasts with the aim of isolating cells secreting antibodies against LDL receptors.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives and Background: A number of neuropathies and myopathies are associated with pathological accumulation of lipids in tissues, including striated muscle. Furthermore, certain genetically transmitted abnormalities are correlated with a gross elevation of fatty acid components normally present only in trace amounts, e.g., that of phytanic acid in Refsum's syndrome or of hexacosanoic acid in adrenoleukodystrophy and adenomyeloneuropathy. Errors in lipid transport, metabolism, and/or synthesis are either known or suspected to be the primary causes of these diseases. The biochemical error associated with Refsum's syndrome had been completely elucidated in the past, mostly through the efforts of investigators in this laboratory, and current studies using skin fibroblasts and striated muscle cells maintained *in vitro* are based, in part, on the experience and knowledge gained in the course of these investigations. This project consisted of several parts, each aiming to study a specific aspect of cholesterol or fatty acid metabolism in human fibroblasts grown in culture. The following studies have been made.

1) Fatty acid metabolism in cells from patients with carnitine deficiency. It has been observed (Engel and Angelini, Science 179: 899, 1973) that skeletal muscles of patients with carnitine deficiency accumulate fat in intracellular droplets. It is also known that carnitine is a cofactor in mitochondrial fatty acid oxidation, and its deficiency is expected to have an impact on their transport and metabolism. We have, therefore, investigated metabolism of fatty acids and the effect of carnitine thereon in normal and carnitine-deficient cells. The clinical research as well as tissue pathology and initiation of cell cultures is being carried out by scientists at NINCDS with whom there is a close cooperation in this study.

2) Metabolism of very long chain fatty acids (C-26) in normal fibroblasts and in those derived from patients with adrenoleukodystrophy and adrenomyeloneuropathy. Increased amounts of very long chain fatty acids were found in tissues of patients suffering from these genetically transmitted neuropathies. It is not known whether these components originate from endogenous or exogenous sources. It has been reported that accumulation of very long chain fatty acids is observed in cultures of fibroblasts derived from patients with these diseases (Kawamura *et al*., Biochem. Biophys. Res. Commun. 82: 114-120, 1978). The study of metabolism, endogenous synthesis and transport of C-26 fatty acid could shed some light on the role of this compound in normal and defective cells. This study is being done in cooperation with scientists of Neonatal and Pediatric Medicine Branch of NICHHD.

3) Effects of steroids on fatty acid oxidation in fibroblasts. This study was an extension of the one reported last year on the effect of steroids on cholesterol synthesis and esterification (project no. Z01 HLB 00624-01 CM).

4) Attempts to prepare monoclonal mouse antibodies to LDL receptors on human fibroblasts. Cells that produce such antibodies could be grown at will

and the antibodies used for assay of LDL receptor activity in human tissues and for studies of the biochemical basis of abnormalities in such activity.

Methods Employed: Human skin fibroblasts or muscle cells derived from control subjects or patients are grown, incubated, and their oxidative activities assayed by standard methods. Monoclonal antibodies to LDL receptors are expected to be produced by hybridomas prepared by fusion of mouse myeloma (P3X63 Ag8) cells with spleen lymphocytes from a mouse immunized with human skin fibroblasts with elevated LDL receptor activity. Medium incubated with these hybridomas is assayed for antibodies against LDL receptors using ^{125}I-protein-A reaction with the immune complex.

Major Findings: 1) It was shown that the assay of the rate of oxidation of 1-^{14}C-palmitic acid is accurate and reproducible when used with skin fibroblasts. With muscle cell cultures, there was larger variability due to some variation between the condition of the replicates. Overnight incubation of confluent cultures of skin fibroblasts with a medium containing 0.5-5 mM L-carnitine stimulated the rate of palmitic acid oxidation by 25-50%. The oxidation was stimulated to a similar extent by carnitine in fibroblasts derived from two patients with adrenomyeloneuropathy and from two patients with systemic carnitine deficiency. In contrast to the L-isomer, D-carinitine was not active. Dexamethasone, which by itself stimulates oxidation of labeled palmitic acid in fibroblasts, increased further the rate of this process when added to medium containing carnitine. This finding is consistent with the known beneficial effects of adrenal corticosteroids on the conditions of patients. Human skeletal muscle cells grown in culture revealed a stimulation by 5 mM carnitine of oxidation of palmitic acid by as much as 100%. Somewhat smaller effect was seen in rat muscle cultures. There was no significant difference between the uptake of ^{14}C-labeled L-carnitine by normal and by carnitine-deficient fibroblasts.

2) A program of study of metabolism of very long chain fatty acids was initiated in cooperation with other investigators. A fair amount of common planning and consultation has been carried out. Laboratory work accomplished in our laboratory consisted of work on organic synthesis of 1-^{14}C-hexacosanoic acid to be subsequently used in metabolic studies.

3) The oxidation of 1-^{14}C-palmitic acid was increased 30-55% in fibroblasts previously incubated for 24 hr with dexamethasone (0.1 to 50 μM). Progesterone and ethinyl estradiol did not affect the rate of fatty acid oxidation.

4) Immune serum of a very high titer was produced in a mouse injected repeatedly with fibroblasts incubated with lipid-depeleted serum and treated with glutaraldehyde. A number of hybridoma lines were obtained after fusion of immune mouse lymphocytes with myeloma cells.

Significance to Biomedical Research: The study of diseases exhibiting abnormalities in fatty acid metabolism may lead to better understanding of their etiology. The study of LDl receptors is centrally important to the metabolism and turnover rates of low density lipoproteins. Study of the effects of corticoids on fatty acid oxidation should increase understanding of basic mechanisms and of therapeutic approaches in diseases of abnormal lipid metabolism.

Proposed Course: 1) It is being planned to study the effect of carnitine on uptake, distribution and storage of exogenous lipids in fibroblasts and muscle cells from patients with carnitine deficiency and from normal controls.

2) Studies of C-26 fatty acid uptake and metabolism in normal and adrenoleukodystrophy cells will be undertaken.

3) The extent and specificity of the glucocorticoid effect on fatty acid oxidation in fibroblasts should be established.

4) More preparative work remains to be done in order to grow cells producing antibodies against LDL receptors.

Publications: Kruth, H.S., Avigan, J., Gamble, W., and Vaughan, M.: Effect of cell density on binding and uptake of low density lipoprotein by human fibroblasts. J. Cell Biol. 83: 588-594, 1979.

Kruth, H.S. and Vaughan, M.: Quantification of low density lipoprotein binding and cholesterol accumulation by single human fibroblasts using fluorescence microscopy. J. Lipid Res. 21: 123-130, 1980.

Annual Report of
Laboratory of Chemical Pharmacology
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

In the past this Laboratory has discovered that several drugs and other foreign compounds cause tissue lesions, such as liver and lung necrosis, through the formation of chemically reactive metabolites. Among these compounds are halogenated benzenes, phenacetin, acetaminophen, isoniazid, iproniazid and furosemide. In some instances, however, the initial chemically reactive metabolite causes toxicities indirectly through the formation of other metabolites. For example, we now believe that some of the mutagenic effects of 2-acetylaminofluorene-N-sulfate are caused by a 2-acetylaminofluorene free radical, which is derived from the nitrenium ion formed from the sulfate derivative. Moreover, the kidney toxicity caused by fluorinated anesthetic gases probably occurs through the formation of unstable intermediates that decompose to fluoride ion, the directly acting nephrotoxic metabolite. Chemically reactive metabolites, however, have been difficult to identify, owing to their chemical instability. Consequently, most of our knowledge concerning their identity is based on indirect evidence. During the past years, a major objective of this Laboratory has been to study the mechanisms by which chemically reactive metabolites are formed and eliminated in tissues and to elucidate basic pharmacokinetic parameters of parent substances and their metabolites that may affect the incidence and severity of the toxicities.

Chemically Reactive Metabolites

Pathways of phenacetin and acetaminophen activation. In the past our Laboratory has shown that the liver necrosis occurring after large doses of these analgesic drugs is mediated by chemically reactive metabolites. However, phenacetin and acetaminophen may be converted to chemically reactive metabolites by several pathways: 1) Phenacetin is converted to phenacetin-3,4-epoxide, which spontaneously loses its ethyl group and reacts with glutathione. 2) Phenacetin is oxidized to N-hydroxyphenacetin, which in turn may be converted to several more active chemically reactive metabolites by several pathways: a) N-Hydroxyphenactin may be converted to phenacetin-N-sulfate or phenacetin-NO-glucuronide, both of which decompose to chemically reactive metabolites and b) N-hydroxyphenacetin is oxidatively O-dealkylated to N-hydroxyacetamiophen, which spontaneously dehydrates to N-acetylimidoquinone, a well-known arylating agent. Details of these mechanisms have been discussed in previous annual reports.

In mammals, phenacetin is metabolized mainly to acetaminophen a small proportion of which is converted to a toxic, chemically reactive metabolite. Although we know that the toxic metabolite is rather short-lived and rapidly reacts with glutathione to form an acetaminophen-glutathione conjugate, the mechanism of its formation remains obscure. Last year we reported evidence that the reactive metabolite of acetaminophen is not derived from either N-hydroxyacetaminophen or acetaminophen-2,3-epoxide. During the past year we

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have considered the possibility that the chemically reactive metabolite is either N-acetylimidoquinone or N-acetylsemiimidoquinone, formed directly from acetaminophen, perhaps by a peroxidase-like mechanism of cytochrome P-450. In support of this view, the covalent binding of ring-labeled acetaminophen in liver microsomal preparations, equalled the sum of the covalent binding of acetyl-labeled acetaminophen and the formation of acetamide which suggests the formation of N-acetylimidoquinone, a part of which becomes covalently bound to protein and a part of which undergoes hydrolysis to acetamide and quinone which also becomes covalently bound. Moreover, both the formation of acetamide and the covalent binding of the reactive metabolite of acetaminophen were blocked by the addition of ascorbic acid or a combination of glutathione and the soluble fraction containing glutathione transferases.

Several years ago we observed that the total amount of chemically reactive metabolite trapped as covalently bound material and the glutathione conjugate increased as the concentration of glutathione was increased. It now seems likely that liver microsomes and NADPH not only convert acetaminophen to the chemically reactive metabolite but also reduce it back to acetaminophen;in this mechanism glutathione would react with the reactive metabolite and thereby increase the amount of trapped metabolite but decrease the amount reduced back to acetaminophen. In accord with this view, glutathione decreases rather than increases acetaminophen-dependent NADPH oxidation by liver microsomes.

We have also demonstrated that a chemically reactive metabolite can be formed from acetaminophen by horseradish peroxidase and hydrogen peroxide. Moreover, acetaminophen almost instantaneously destroys complex II, the free radical intermediate of the horseradish peroxidase-H_2O_2 system. Although these findings indicate that acetaminophen can be oxidized by a series of one electron oxidation steps, we were unable to demonstrate a complex II with cytochrome P-450 in the presence of either NADPH or cumene hydroperoxide. Thus the peroxidase mechanism remains doubtful.

Metabolism of halogenated alkanes and alkyl ethers. Rat liver microsomes convert deuterated chloroform ($CDCl_3$) to phosgene about 50% as rapidly as they convert chloroform ($CHCl_3$) to phosgene. During the past year we have used the isotope effect to study the cytochrome P-450 dependent metabolism of halogenated alkyl ethers used as anesthetic gases. For this purpose we devised a method for the synthesis of the three deuterated forms of enflurane, namely 1) CDF_2OCF_2CHClF, 2) CDF_2OCF_2CDClF and 3) CHF_2OCF_2CDClF. With these forms we were able to show that rat liver microsomes catalyze the release of fluoride ion almost exclusively from the CHClF group of enflurane; the release of fluoride from forms 3 and 4 occurred about 25-30% as rapidly as from enflurane and form 1. Moreover, we were able to prove that rat liver microsomes convert enflurane almost exclusively to difluoromethoxydifluoroacetic acid ($CHF_2OCF_2CO_2H$); there was no evidence for the formation of chlorofluoroacetic acid, a substance that should have been formed if enflurane were hydroxylated at the CHF_2 group. By contrast, rat liver microsomes also catalyze the slow release of fluoride ions from isofluorane ($CHF_2OCHClCF_3$) but not from either $CHF_2OCCl_2CF_3$ or $CHF_2OCF_2CFCl_2$. These

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findings suggest that isofluorane is first converted to $CHF_2OC(OH)\ ClCF_3$, which would decompose spontaneously to formic acid, trifluoroacetic acid, fluoride and chloride ions.

In confirmation of Rice et al., treatment of rats with isoniazid or hydra-azine markedly increases the rate of metabolism of enflurane by liver microsomes. Indeed the rate of fluoride release from enflurane by liver microsomes from rats pretreated with isoniazid can be as much as 10-fold greater than the release by liver microsomes from rats pretreated with phenobarbital. The mechanism for the increase in the activity of the cytochrome P-450 that catalyzes the oxidative dehalogenation of enflurane, however, is unusual; pretreatment of rats with isoniazid does not cause an increase in the total amount of cytochrome P-450 in liver microsomes, nor does it cause any obvious change in the relative amounts of the various forms of cytochrome P-450 as determined by polyacrylamide gel electrophoresis. Moreover, the reconstitution of purified cytochrome P-450 systems of liver microsomes from isoniazid pretreated rats result in the same activity as that from control rats. Thus, the mechanism of "induction" caused by isoniazid is obscure.

Studies with 3 patients undergoing enflurane anesthesia have revealed the presence of difluoromethoxydifluoroacetic acid (90-470 μmoles) but not of chlorofluoroacetic acid in urines collected for 15-24 hrs after anesthesia. Thus, the major pathway of enflurane metabolism in rats and humans is the same. Although the amount of fluoride formed from enflurane ordinarily is not sufficient to cause kidney damage, it is possible that patients receiving "inducers" such as isoniazid, may be at risk when subjected to prolonged anesthesia.

In rats pretreated with phenobarbital, $CHCl_3$ decreases the concentration of glutathione in liver. Studies during the past year have shown that the concentration of glutathione in liver of rats pretreated with phenobarbital is also decreased by the administration of trichlorobromomethane, bromoform and carbon tetrachloride, the relative potencies being $CHCl_3 > CBrCl_3 > CHBr_3 = CCl_4$. Moreover, all four compounds were converted to a biliary metabolite subsequently identified by nuclear magnetic resonance spectroscopy as GS-CO-SG. These results indicate that the decrease in glutathione in the liver is probably due to phosgene formed from all four compounds. The hepatotoxocity of CCl_4 and $CBrCl_3$ cannot be due solely to phosgene, however, because these substances are more toxic than either $CHCl_3$ or $CHBr_3$.

Chloramphenicol metabolism. Chloramphenicol undergoes several unusual metabolic transformations in the liver. For example, in the presence of glutathione the soluble fraction of liver converts chloramphenicol to two metabolites, one of which is chloramphenicol aldehyde and the other of which is a chloramphenicol oxamyl derivative. During the past year, we have demonstrated that chloramphenicol is converted to the aldehyde by only one of the glutathione transferases isolated by Jakoby, namely enzyme A. This enzyme also catalyzes the dechlorination of thiamphenicol (the methyl sulfonyl analog of chloramphenicol) but does not catalyze the dehalogenation of halothane or enflurane. In turn chloramphenicol aldehyde is oxidized by another enzyme

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in the soluble fraction that requires NAD or NADP.

Several years ago we found that liver microsomes in the presence of NADPH and oxygen converted chloramphenicol to chloramphenicol oxamic acid, presumably through the formation of a hydroxydichloromethyl group. We now have found that under aerobic conditions liver microsomes convert chloramphenicol to 5 other minor metabolites, one of which is p-nitrobenzyl alcohol; the ability of cytochrome P-450 to catalyze this kind of cleavage has never been before shown. Moreover, under anaerobic conditions liver microsomes convert chloramphenicol to its monochloro analog, presumably by a reductive dechlorination reaction analogous to the conversion of carbon tetrachloride to chloroform.

The finding that the aplastic anemia caused by chloramphenicol in cattle could not be entirely prevented by the administration of a vitamin B complex raised the possibility that the aplastic anemia occurring in the cattle receiving the vitamin supplement might be due to one of the nitro reduction metabolites of chloramphenicol formed by bacteria in the rumen. However, the nitroso analogue of chloramphenicol failed to cause aplastic anemia either in cattle or in rats. These findings probably also preclude the possibility that the aplastic anemia is caused by the hydroxylamine analogue, because xanthine oxidase, ubiquitous enzyme in mammals, rapidly reduces the nitroso compound to its hydroxylamine metabolite.

Pharmacokinetics of drugs and their metabolites. Since many substances exert pharmacologic and toxicologic actions through the formation of their metabolites, it is important to understand the basic concepts of the pharmacokinetics of metabolite formation and elimination by first order mechanisms and to evaluate how various factors can alter their pharmacokinetic parameters. For example, pharmacokinetic equations predict that when a drug is eliminated from the body by first order systems in a single organ, the area under the curve (AUC) of the plasma concentration of a metabolite of the drug will be independent of the route of administration of the drug. Thus the finding that the AUC of a metabolite differs with the route of administration suggests either that the drug is eliminated from the body by two or more organs or that one or more mechanisms of elimination of the drug in an organ does not follow first order kinetics. Thus the determination of the AUC of a drug metabolite after different routes of metabolism is useful in alerting investigators to the occurrence of unusual pharmacokinetic mechanisms.

Simultaneous elimination by liver and kidney. As pointed out last year, when a drug is eliminated by both the kidney and the liver the ratio of the AUC values of a metabolite formed solely by the liver after a given dose is administered orally and intravenously should be $(1 + Cl_R/Q_H)$; where Cl_R is the renal clearance and Q_H is the total blood flow rate through the liver. To demonstrate this relationship we studied the biliary excretion of radiolabeled p-aminohippurate under steady state conditions during the simultaneous infusion of ^{14}C-PAH intraportally and ^{3}H-PAH intravenously into rats. The ratio $^{14}C/^{3}H$ excreted into bile under steady-state conditions in various rats ranged from 1.20-1.56. By measuring the renal clearance of

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PAH, we were then able to calculate that the hepatic blood flow rate in the animals ranged from 40-80 ml/kg/min. The principle illustrated in these studies may be used to develop relatively simple methods for evaluating changes in hepatic blood flow rates which occur in diseases such as cirrhosis, or with cardiovascular drugs. For this purpose, we are searching for a drug that is eliminated from the body solely by conversion to metabolite I, which in turn is both rapidly excreted by the kidney and metabolized to metabolite II in the liver.

Pharmacokinetics of the metabolites of tricyclic antidepressants. Both amitriptyline and imipramine are converted to hydroxylated N-demethylated metabolites either by N-demethylation followed by hydroxylation or by hydroxylation followed by N-demethylation. In collaboration with the Karolinska Institute in Stockholm, Sweden, we discovered that the N-demethylation metabolite nortriptyline (NT) appears in blood of patients receiving amitriptyline orally but not after receiving the drug intramuscularly. Since the AUC values of NT should have been the same after the two routes of administration if the drug were metabolized solely by the liver by first order reactions, it became evident that the pharmacokinetics of amitriptyline is unusual in patients. During the past year studies were undertaken to determine: 1) whether a similar effect occurs with imipramine, which is being studied by W.Z. Potter, NIMH and 2) whether the rat might serve as a suitable animal model for studying and differences in imipramine metabolism found in humans.

In an attempt to assess the effects of high drug concentrations in the portal vein that presumably occur during the absorption of orally administered drugs on the pattern of imipramine metabolism, tracer doses of radiolabeled imipramine were administered intramuscularly to two groups of rats and a large dose of unlabeled imipramine was administered orally to one of the groups. Strangely, however, the amount of radiolabeled material excreted as the hydroxy-N-desmethylimipramine was greater and the amount excreted as the N-desmethylimipramine was smaller in the group of rats given the oral dose of unlabeled imipramine than in the group that didn't. Thus, the clearance of N-desmethylimipramine appeared to be smaller after low doses of imipramine than after high doses of the drug. In vitro studies revealed that at low concentrations desmethylimipramine leads to the formation of an inactive complex of cytochrome P-450. Moreover, the formation of the complex does not occur with high concentrations of N-desmethylimipramine and is blocked by imipramine. Under these conditions, the clearance of desmethylimipramine would appear to be smaller after small doses than after large doses of imipramine.

Pharmacokinetics of cephaloridine excretion. The renal clearances of cephaloridine and cephapirin in humans decrease as their concentrations in blood decrease, suggesting the presence of an active transport system in kidney tubules that mediates the reabsorption of the drugs (Arvidsson et al. Clin. Pharmacol.Ther. 25:870,1979). In attempting to determine the normal role of the reabsorption transport system, we discovered that cephaloridine in rats increases the clearance of uric acid. Whether cephaloridine also acts as an uricosuric drug in humans remains to be determined.

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Stereospecific metabolism of oxyprenolol. Since this β-adrenergic blocking agent contains an asymmetric carbon, it exists as R and S forms which may be metabolized at different rates by cytochrome P-450 enzymes by liver microsomes. Studies during the past year have revealed that there are marked species differences in the relative rates the R and S forms are metabolized by liver microsomes by the major reactions, namely O-dealkylation, N-dealkylation and ring hydroxylation. Presumably these species differences are due to differences in the substrate specificity and position specificity of the cytochome P-450 enzymes in liver microsomes.

Mechanisms of Toxicity and Drug Action

Effects of Ca^{++}-induced toxicity. Although it is well established that CCl_4 causes liver necrosis through the formation of chemically reactive metabolite, the mechanism in which the toxic metabolite causes cellular death is not clearly established. During the past year, we found that calcium and the ionophore A 23187 does not alter the formation of the chemically reactive metabolite by isolated hepatocytes as measured by covalent binding of the toxic metabolite. Nevertheless, we find the presence of 3.6 mM Ca^{++} markedly potentiates the toxicity of CCl_4 as determined by a series of criteria, including the release of lactic acid dehydrogenase and the formation of "blebs" as revealed by scanning electron microscopy. Thus Ca^{++} provides a tool to help us elucidate the critical events that lead to cellular damage. Whether the effects of Ca^{++} on cellular damage caused by other toxicants remains to be determined.

Analogs of anacardic acid as inhibitors of prostacyclin synthesis. Anacardic acid isolated from cashew nuts comprises a group of salicyclic acid analouges containing C_{15} aliphatic side chains having various numbers of double bonds. During the past year we found that only the analogs containing 2 or 3 double bonds could inhibit prostacyclin synthesis in human platelets and thereby inhibit platelet aggregation or could serve as mollusicides. The triene was about twice as active as the diene. Perhaps even more potent analogues may be developed by altering the structure of these substances.

SAR of several analogs of prostacyclin. The potency of prostacyclin in preventing platelet aggregation is not changed by lengthening the side chain to form 20-methyl prostacyclin but is almost abolished by shortening it to 20-normethyl prostacyclin. However, the introduction of an acetylenic group into 20-methyl prostacyclin to form to 20-methyl (13,14-didehydro) prostacyclin increases the potency of prostacyclin. Introduction of a methyl group at the C-16 position decreased the potency and replacement of the oxygen by a methylene group abolished the activity. Similar effects were obtained when PGI_2 activity was tested on platelet membrane adenyl cyclase.

Antibodies against guanylate cyclase. In order to determine in localization of this enzyme in various tissues we have prepared antibodies against the enzyme isolated from sea urchins in both mice and rabbits. We are now attempting to form monoclonal antibodies by hybridoma techniques.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00810-03 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Kinetics of Formation and Elimination of Drug Metabolites by the Liver

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrence J. Monks	Vist. Fellow	LCP	NHLBI
OTHER:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A method for the quantification of imipramine and its metabolites has been developed using HPLC. Utilizing this assay we studied the kinetics of imipramine metabolism in rats and in rat liver microsomes. The in vivo studies suggested that the conversion of desimipramine to hydroxy desimipramine after intramuscularly administered ^{3}H-imipramine occurs more rapidly when oral unlabeled imipramine is also administered than when it is not. Studies with rat liver microsomes suggest that low doses of imipramine lead to an inactive cytochrome P-450 complex that is not formed when large doses of imipramine are administered.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: After the oral administration of amitriptyline in man a major fraction of that drug is demethylated to nortriptyline while after intramuscular administration the concentration of this metabolite in plasma was below the limit of detection. These findings suggest that during oral administration of amitriptyline the concentration of the drug reaches high enough concentrations to saturate the enzyme that catalyzes the hydroxylation of the drug and its demethylated metabolite nortriptyline.

The purpose of the present study was to determine whether a similar kind of mechanism occurs during the metabolism of imipramine in rats.

Methods Employed: It was necessary to develop a sensitive and specific assay for imipramine and its metabolites in biological samples. The method depends on extraction of the samples with hexane followed by high pressure liquid chromatography and fluorometric detection. This method permits quantification of imipramine and its metabolites down to the 1 ng/ml level.

The kinetics of C-hydroxylations and N-demethylation of imipramine and of the C-hydroxylation of desipramine were studied in rat liver microsomes. The binding of imipramine and desipramine to cytochrome P-450 was studied spectrophotometrically. The effects of an oral dose of imipramine on the fate of an intramuscular tracer dose of ^{3}H-imipramine was investigated.

Major Findings: The oral administration of unlabeled imipramine to rats receiving a tracer dose of ^{3}H-imipramine intramuscularly did not appreciably affect the excretion of radiolabeled imipramine or hydroxy imipramine, when compared with the excretion of these substances by rats receiving a tracer dose of ^{3}H-imipramine intramuscularly alone. Instead, it decreased the excretion of desipramine and increased the excretion of hydroxy desipramine. Thus, the oral dose appeared to enhance the conversion of desipramine to hydroxy desipramine in rats.

The kinetics of imipramine metabolism by liver microsomes would not have predicted the *in vivo* results. The K_m's for the hydroxylation and demethylation of imipramine by rat liver microsomes were 7.4 µM and 28.0 uM, respectively, the maximum velocity of the reactions being 1.64 nmol/mg/min and 5.5 nmol/mg/min. Desipramine was shown to be a competitive inhibitor of imipramine hydroxylation. The parameters for desipramine hydroxylation were K_m, 10.8 uM, V_{max} 0.16 nmol/mg/min. Hydroxy imipramine was also shown to be further metabolized to hydroxy desipramine as determined by the Nash assay with a K_m 7.2 uM and V_{max} 2.0 nm/mg/min. Higher concentrations of microsomes were found to give an increase in the apparent K_m values for both imipramine hydroxylation and demethylation. This decrease in specific activity could be caused by binding of the drug to nonspecific sites on microsomes.

Binding studies with imipramine and desipramine revealed that they both elicit type I binding spectra and that they have identical binding constants (2 μM). Studies also indicate the formation of an inactive cytochrome P-450 complex when NADPH is added to the sample cuvette in the presence of substrate K_m concentrations of desipramine. However, the complex does not not form in the presence of large concentrations of desipramine and imipramine. The data are thus consistent with the view that the inactive complex is formed during the metabolism of tracer doses of ^{3}H-imipramine but not after large oral doses of unlabeled drug.

Significance to Biomedical Research and Program of the Institute: The extent of demethylation of imipramine may be clinically important since the metabolite formed is pharmacologicaly active. It is, therefore, important to recognize any route related differences in the formation of this metabolite and to elucidate the mechanisms responsible.

Proposed Course of Project: We have shown that isolated rat liver hepatocytes can metabolize imipramine to its two major metabolites extensively. We intend to compare the kinetics of imipramine metabolism in isolated hepatocytes and liver microsomes.

In view of the apparently considerable enterohepatic circulation of imipramine metabolites we intend to study imipramine metabolism in bile-cannulated rats utilizing different oral doses.

The desipramine/cytochrome P-450 complex is to be investigated further.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00831-05 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Chloramphenicol-induced Aplastic Anemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Siegel	Guest Worker	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI
OTHER:	Upton Talley	Animal Technician	DIR.	NHLBI

COOPERATING UNITS (if any)

Upton Talley is an Animal Technician from the Office of the Director, Division of Intramural Research, NHLBI.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.6	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of chloramphenicol's reductive metabolism in chloramphenicol-induced aplastic anemia is being studied. In vitro studies of chloramphenicol was shown to be reduced to amine by rat cecum contents. However, xanthine oxidase failed to reduce chloramphenicol however, it converted nitroso chloramphenicol to N-hydroxy chloramphenicol indicating different enzyme systems for the reduction for chloramphenicol. In in vivo studies nitroso chloramphenicol failed to produce aplastic anemia in mice at doses up to 40 mg/kg i.p.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The main objective of this study is to elucidate the mechanism by which chloramphenicol induces aplastic anemia. Thiamphenicol an analog of chloramphenicol without the aromatic nitro group has not been reported to cause aplastic anemia. It, therefore, has been suggested that chloramphenicol's toxicity is through its reductive metabolism of the aromatic nitro group. The initial objectives of this work are to characterize various routes of reduction of chloramphenicol and the identification of intermediate metabolites. These metabolites will be tested for their ability to produce aplastic anemia in mice as well as bovine since we have shown earlier that chloramphenicol induces aplastic anemia when administered orally in Holstein calves.

Methods Employed: Nitroso chloramphenicol and N-hydroxy chloramphenicol were synthesized according to published procedures. Chloramphenicol amine was synthesized by coupling the reduced chloramphenicol base with methyl dichloroacetate.

Enzymatic reduction of ^{14}C-chloramphenicol and ^{14}C-nitroso chloramphenicol were followed using either rat cecum contents or purified xanthine oxidase. Enzyme reactions were carried out under a nitrogen atmosphere. Products were separated by thin layer chromatography and quantitated by liquid scintillation counting. The enzyme reduction of nitroso chloramphenicol was also monitored by spectrophotometry.

Mice and calves were injected daily with nitroso chloramphenicol for 10 days and were followed for 6 week after the final injection. Once or twice a week blood samples were analyzed for red and white blood cell and hemaglobin content. At the end of the test period the animals were sacrificed and various tissues including bone marrow were taken for histopathological examination after fixation and staining.

Major Findings: Chloramphenicol as expected was readily reduced by rat cecum contents. Xanthine oxidase as reported earlier by this Laboratory failed to reduce chloramphenicol. However, xanthine oxidase readily reduced nitroso chloramphenicol. This reaction was partially inhibited by oxygen. In the NIH all purpose strain of mice, the nitroso chloramphenicol toxicity study showed no toxic symptoms during the 10 days of injection except at high doses (40 mg/kg) which induced irritation of the intraperitoneal cavity. During the following 6 week period, the mice showed no dose dependent changes in their blood cell count. Upon termination of the study, necropsy of the animals revealed a dose dependent relationship in formation of peritoneal adhesions. Initial histopathological studies indicate no significant change in any bone marrow cells.

In the bovine calves the nitroso chloramphenicol so far have shown no apparent symptoms during 10 days of intravenous injections of the drug at 2.5 mg/kg/day. During the follow up period there has been no indications of aplastic anemia from the measurement of peripheral blood cell counts. From these toxicity data nitroso chloramphenicol may not be considered a potent aplastic anemia inducing agent *in vivo*.

Significance to Biomedical Research and the Program of the Institute: The major finding that nitroso chloramphenicol can be readily reduced by xanthine oxidase even in the presence of oxygen raises the possibility that N-hydroxylamine or a free radical intermediate in the reduction step may play an important role in the chloramphenicol aplastic anemia.

Proposed Course of Project: We propose to terminate this project.

Publications:

Pohl, L.R., Reddy, G.B. and Krishna, G.: A new pathway of metabolism of chloramphenicol which influences the interpretation of its irreversible binding to protein *in vivo*. *Biochem. Pharmacol.* *28*: 2433-2440, 1979.

Gaion, R. and Krishna, G.: A possible role for calcium and cyclic GMP in hormone-induced lipolysis. Presented at the International Symposium on Obesity: Pathogenesis and Treatment, Jan. 17-18, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00834-05 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cyclic 3'5' Cytidine Monophosphate

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gopal Krishna	Chief, Section	LCP	NHLBI
OTHER:	Thomas Hundley	Biol. Lab. Tech.	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.3	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Last year we reported the development of a very sensitive radioimmunoassay for cyclic 3'5'cytidine monophosphate. Utilizing this method in combination with ion-exchange chromatogaphy on Dowex 1 columns we have clearly demonstrated that cyclic CMP does not exist in various tissues or biological fluids. Further we had shown that the rat pancreas which appeared to contain some apparent cyclic CMP is probably due to cyclic 2'3'-CMP which is formed from RNA. So the existence of cyclic CMP or its role in cell proliferation is highly questionable.

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Project Description:

Objectives: Last year we reported that we were unable to detect cyclic CMP in rat liver, human urine or mouse leukemia L1210 cells. Rat pancreas appeared to be the only tissue that contained low amounts of cyclic CMP in the range of 0.5 - 1.0 pmole/g. One of the main objectives of this study is to reconfirm the absence of cyclic CMP in various tissues and biological fluids, and to characterize the presence of cyclic CMP in pancreas is only due to 2,3-cyclic CMP and not to any other nucleotide.

Methods Employed: A radioimmunoassay of cyclic AMP was carried out as described in last year's report. The tissue extracts were chromatographed on Dowex 1-formate and the columns were eluted with increasing concentrations of formic acid. Assays for cyclic CMP were carried out on all fractions (2 ml) eluted from the columns.

Major Findings: So far we have been unable to detect the presence of cyclic CMP in tissues such as liver, spleen, mouse leukemia L1210 cells, human urine except rat pancreas. The sensitivity of the radioimmunoassay is such that we could have detected the presence of at least 10-20 pmoles of cyclic CMP. As we reported last year we were able to confirm again the presence of an apparent cyclic CMP in the range of 1-3 pmoles/g of rat pancreas. However the elution profile in Dowex-1 chromatography did not correspond exactly to the authentic cyclic CMP. The elution profile corresponded more to authentic cyclic 2'3'-cytidine monophosphate. This nucleotide has been shown to be formed by degradation of RNA. Even though cyclic 2'3' CMP did not corss react to a great extent with the specific cyclic CMP antiseria we had employed in our assays, it had sufficient cross reactivity to interfere with cyclic CMP assay. When the Dowex-1 column was eluted with lower concentrations of formic acid, it was possible to separate cyclic CMP from other interferring nucleotides in pancreas such as cyclic 2'3' CMP,2'CMP and 3'CMP. There was no detectable cyclic CMP in any fraction. We conclude, therefore, the presence of cyclic CMP reported in pancreas and other tissues are probably due to interference from other nucleotides rather than cyclic CMP.

Significance to Biomedical Research and the Program of the Institute: Cyclic CMP may not be present in any tissue or biological fluids so far examined, and its role in tissue proliferation thus remains highly questionable.

Proposed Course of Project: We will terminate this project.

Publication: Kaushal, D.C, Carter, R., Miller, L.H. and Krishna, G.: Gametocytogenesis by malaria parasites in a continuous culture: Nature, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00837-04 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

On the Mechanism of Hydrolytic Dechlorination of Chloramphenicol

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie L. Martin	Biologist	LCP	NHLBI
	Brion Gross	Costep	LCP	NHLBI
	Patricia Morris	Biologist	LCP	NHLBI
	John George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

William B. Jakoby is in the Section on Enzymes and Cellular Biochemistry, National Institute of Arthritis, Metabolism and Digestive Diseases

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.6	PROFESSIONAL: 0.2	OTHER: 1.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Chloramphenicol (CAP, $RNHCOCHCl_2$) is dehalogenated to CAP aldehyde (RNHCOCHO) in rat cytosol predominantly by glutathione transferase A. This reaction likely involves the initial displacement of a chlorine by glutathione (GSH) to produce an alpha halothioether intermediate (RNHCOCHCl-SG). This product is expected to react rapidly with water to yield CAP aldehyde (RNHCOCHO) and GSH. Thiamphenicol, TAP, the p-methylsulfonyl derivative of CAP, is dechlorinated to an aldehyde by this enzyme system to a significantly slower extent than is CAP. In contrast, neither enflurane (CF_2OCF_2CHClF) nor halothane ($CF_3CHBrCl$) is dehalogenated to aldehydes by this enzyme system. This finding suggests that the GSH dependent dehalogenase are not involved in the liver and kidney toxicity produced by these compounds.

Project Description:

Objectives: We previously reported that chloramphenicol (CAP, $RNHCOCHCl_2$) is metabolized into CAP aldehyde (RNHCOCHO) and CAP oxamic acid (RNHCOCOOH) by a glutathione (GSH) dependent enzyme or enzymes in the 100,000 x g supernatant (cytosol) of rat liver. The objective of this investigation has been to characterize more completely the enzyme(s) involved in these biotransformations.

Methods Employed: The metabolites were assayed by high pressure liquid chromatography (HPLC), and their structures were determined by isobutane chemical ionization mass spectrscopy. Purified preparations of rat GSH transferase were supplied by Dr. William Jakoby.

Major Findings: CAP was metabolized into CAP aldehyde by purified GSH transferases in the presence of GSH. Enzyme A was the most active, whereas enzymes AA, B, and C showed much less activity. These enzymes, however, did not metabolize CAP nor CAP aldehyde into CAP oxamic acid. Another enzyme(s) in the cytosol of rat liver was responsible for the conversion of CAP aldehyde into CAP oxamic acid. This enzyme(s) utilized NAD or NADP as a cofactor, but did not require GSH.

Thiamphenicol (TAP), the methylsulfonyl derivative of CAP ($CH_3SO_2C_6H_4$) was also dehalogenated to from an aldehyde derivative by the cytosol of rat liver, although at approximately 30% of the rate of that of CAP. In contrast, neither enflurane (CHF_2OCF_2CHClF) nor halothane ($CF_3CHBrCl$) were dehalogenated into aldehyde derivatives by the cytosol enzymes.

Significance to Biomedical Research and Program of the Institute: These results strongly indicate that GSH transferase A is predominantly responsible for the dechlorination of CAP to CAP aldehyde in the cytosol reaction of liver. A potential mechanism for this reaction involves the initial displacement of a chlorine by GSH to produce an alpha-halothioether intermediate (RNHCOCHCl-SG). This product would be expected to hydrolyze rapidly to yield CAP aldehyde (RNHCOCHO) and GSH. CAP aldehyde is then likely metabolized to CAP oxamic acid (RNHCOCOOH) by nonspecific aldehyde oxidase present in the cytosol of liver.

The observation that neither enflurane nor halothane is dehalogenated by the cytosol of rat liver, suggests that this pathway of metabolism is not involved in the liver and kidney toxicities associated with the administration of these drugs.

Proposed Course of Project. This project is terminated.

Publications:

Martin, J.L., George, J.W., and Pohl, L.R.: Glutathione-dependent dechlorination of chloramphenicol by cytosol of rat liver. *Drug Metabolism and Disposition* *8*: 93-97, 1980.

Pohl, L.R., Reddy, G.B. and Krishna, G.: A new pathway of metabolism of chloramphenicol which influences the interaction of its irreversible binding to protein *in vivo*. *Biochem. Pharm.* *28*: 2433-2440, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00843-01 LCP

PERIOD COVERED
October 1, 1979 to October 1, 1980

TITLE OF PROJECT (80 characters or less)
Separation of Rat Hepatocytes by a Coil Planet Centrifuge

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Siegel	Guest Worker	LCP	NHLBI
	Richard Chenery	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. Y. Ito, Lab. of Technical Development, NHLBI

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A method to separate isolated hepatocytes into various cell types was investigated. A preliminary study has indicated that it is possible to separate viable and nonviable cells using a nonsynchronous flow through a coil planet centrifuge. These viable cells retain their cytoplasmic enzymes and drug metabolizing ability even after three hours in the centrifuge.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Various studies have indicated that liver is made up of cell populations which differ markedly in drug metabolism. Separation of these cell populations may be advantageous for the study of compounds which induce differential damage to liver cells. Using a nonsynchronous flow through coil planet centrifuge, we have attempted to separate isolated hepatocytes obtained from phenobarbital induced rats into distinct populations and to study the capacity of these cells to metabolize drugs.

Methods Employed: The isolation of hepatocytes has been described earlier. The cells in normal culture media were subjected to a nonsynchronous flow through a coil planet centrifuge (Model 1, no seal); the flow rate and centrifugal force were used as separation parameters.

Major Findings: Hepatocytes retained in this system for up to three hours were still viable when removed. The system was useful in separation of nonviable from viable cells. The viable cells retained cytoplasmic enzymes (lactate dehydrogenase) and drug metabolizing ability (chloramphenicol glucuronic acid conjugation). Nonviable cells did not retain any of these functions.

One of the problems that was encountered in the separation of populations of viable cells had been clumping of cells during the centrifugation. Several methods to overcome this problem has met with only partial success.

Significance to Biomedical Research and Program of the Institute: Separation of different cell types from isolated rat liver cells will be of great importance in the study of the mechanism of action of different hepatotoxins which affect the cells located in the centrilobular area from those located in the midzonal and periportal area of the liver.

Proposed Course of Project: We are presently attempting to find ways to prevent clumping of cells and then to examine whether this method will be useful in the separation of various cell types in the liver and extend the methodology to other cells such as lung.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 00844-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Anacardic Acid: SAR for Molluscicidal Action and Platelet Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gopal Krishna	Chief, Section	LCP	NHLBI
OTHERS:	Nancy Kim	Chemist	LCP	NHLBI
	John T. Sullivan	Vist. Fellow	LP	NIAID
	Helen Lloyd	Chemist	LC	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Anacardic acid analogs have been tested as inhibitors of prostacyclin (PGI_2) induced cyclic AMP synthesis in human platelets as well as on their molluscicidal action against B. glabrata. Triene analog of anacardic acid was found to be more potent than diene and monoene analogs in both platelets and snails. The decarboxylated derivative of anacardic acids and salicylic acid were inactive as molluscicides. It appears that both salicylic acid and the unsaturated side chain plays an important role for the biological action of anacardic acids.

Project No. Z01 HL 00844-01 LCP

Project Description:

Objectives: Last year we reported on the isolation and characterization of anacardic acids obtained from cashew shell. Anacardic acids are a mixture of salicylic acid analogues containing 15 carbon aliphatic side chain in various degrees of unsaturation. Some of the analogues of anacardic acid was found to be an inhibitor of prostacyclin action in human platelets and also were found to have potent molluscicidal effect when tested against fresh water snails since the snails are potential vectors for Schistosomioses. The understanding of the molluscicidal action of anacardic acid may not only help in the development of anacardic acids as new class of naturally occcurring mollusicide but may also reveal the interrelationship between prostacyclin action in the platelets as well as in the snails. The main objective of this study has been to characterize the structural requirements for both molluscicidal action as well as on the inhibitory effect of anacardic acid on prostacyclin induced cyclic AMP in human platelets.

Methods Employed: Various analogues of anacardic acid were isolated from Cashew shell by high pressure chroamtography. All analogues were characterized by a number of techniques including gas chromatography and mass spectrometry.

Prostacyclin (PGI_2) induced cyclic AMP was measured in human blood platelets as reported earlier and the effect of various analogs of anacardic acid on PGI_2 induced cyclic AMP synthesis was determined.

One of the genetic stocks of snail, Biomphalaria glabralaa (NIH albino or M Line) was utilized for molluscicidal testing. Approximately 100 breeding snails were maintained in artificial pond water and were fed Romaine lettuce. Eggs were collected from these snails at weekly intervals and were allowed to hatch in fresh pond water. The newly hatched snails were transferred to an aquarium and were fed lettuce, dried dog food pellets and and calcium carbonate until they obtained 5-7 mm shell diameter. Ten snails were transferred into a polyethylene bag containing 1 liter of deionized water to which concentrated solution of anacardic acid in methanol (up to 1 ml) had been added to attain various concentrations of anacardic acid. The polyethylene bag was tied so that no large air bubbles remained and the bag was placed in an incubator at 26.5°C for 24 hr. At the end of incubation the snails were removed and examined under binocular dissection microscope for heartbeats. Control snails were treated in a similar manner except no anacardic acid was used. Methanol used in these experiments did not have any effect. Mortality data were analyzed with the probit and potency analysis program devised by Daum (Bull. Ent. Soc. Amer. 16:10,1970).

Major Findings: The triene analogue of anacardic acid appears to be the most potent inhibitor of prostacyclin-induced cyclic AMP synthesis in human blood platelets in comparison with the other analogues. The diene analogue was more potent than the monene and the saturated analogue was without any effect.

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The triene an analogue was found to be the most potent molluscicide (triene, LD_{50} - 0.8 ppm vs diene, LD_{50} - 1.4 ppm) and the saturated analogue was not effective as a molluscicide at concentrations up to 5 ppm. The decarboxylated derivatives of anacardic acid (cardanol) were devoid of molluscicidal action at concentrations up to 15 ppm. Salicyclic acid was not active as a mollusicide. These results indicate that this requirement of the unsaturated side chain as well as the carboxyl group of salicyclic acid in the anacardic acid molecule plays an important role in the biological effect. We have also shown a simple methanolic extract of Cashew shell can be utilized for controlling the snail population and its molluscicidal action is mainly due to the presence of the unsaturated analogues of anacardic acid. This was revealed by testing all fractions eluting from high pressure liquid chromatography of the crude extract of Cashew shell; only fractions containing anacardic acids (monoene, diene and triene) were active as molluscicide.

Significance of Biomedical Research and Progaram of the Institute: The discovery of the molluscicidal action as well as the anti PGI_2 action of anacardic acid is due to the unsaturated aliphatic chain and the salicylic acid moeity may help in the understanding of the mechanism that may be involved in the killing of the snails. If PGI_2 plays an important role in the maintaining of snail life it may be possible to test other compounds which may be more potent in inhibiting PGI_2 action as a potential molluscicides.

Proposed Course of Project: We propose to modify the anacardic acid molecule in order to develop a more potent inhibitor of PGI_2 action as well as more potent molluscicides.

Publications:

Lloyd, H.A., Denny, C. and Krishna, G.: A simple liquid chromatography method for analysis and isolation of unsaturated components of anacardic acids. Liquid Chromatography, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00845-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Guanylate cyclase: preparation of monoclonal and polyclonal antibodies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gopal Krishna	Chief, Section	LCP	NHLBI
OTHER:	Thomas Hundley	Biol. Lab. Tech.	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.3	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

During the past year we have been able to purify large quantities of guanylate cyclase from sea urchins. The purified enzyme showing a single band in gel electrophoresis was able to convert 16 umole min^{-1} mg^{-1} GTP to cyclic GMP. The enzyme appears to contain 2 subunits of apparent M.W.59000. After repeated injections of purified enzyme in rabbits over a period of one year, the sera contained a specific antibody against guanyalte cyclase. In mice, the antibody appears to be produced within 2 months of injection. The antisera showed a very discrete single precipitin line against purified as well as partially purified guanylate cyclase. It also inhibited guanylate cyclase. The production of monoclonal antibody is under progress.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: We have shown earlier that cyclic GMP plays an important role in various secretory functions. Moreover, we have shown that cyclic GMP as well as the enzymes involved in its synthesis and degradation are highly concentrated in the rod outer segments of the retina. Even though the exact role of cyclic GMP in the vision process is not clearly understood it appeas to be involved in the degeneration of rods. We have shown earlier that in Irish setter dogs which have inherited rod-cone dysplasia, cyclic GMP is markedly increased before the degeneration of both rods and cones. The enzyme guanylate cyclase, which catalyzes in the synthesis of cyclic GMP, appears to be present in the rod-outer segments of the retina but its exact location is not known whether the enzyme is present in other tissues such as pancreas, platelets, lung and fat cells is also not known. In order to visualize the enzyme by histo-immunochemical techniques it is highly imparitive to develop specific antibodies against guanylate cyclase. The main objective of this study has been to purify guanylate cyclase and to prepare antibody against this enzyme by both conventional technique in rabbits and mice and to prepare monoclonal antibodies with specific characteristics using hybridomas techniques.

Methods Employed: Guanylate cyclase was purified from sea urchin (Arbacia punculata) sperms. The membrane bound enzyme was solubilized by 1% Lubrol in 25 mM triethanolamine buffer (pH 7.6) containing 2 mM dithioerythrotol. The enzyme was purified by sequential chromatography in GTP sepharose, DEAE sephadex and bio-gel (A-0.5) columns. The purified enzyme was characterized by SDS polyacrylamide gel electrophoresis.

Purified guanylate cyclase mixed with complete Freud's adjuvant was injected for one year into rabbits and for two months into mice. The presence of guanylate cyclase antibodies in the sera were detected by immuno diffusion in Ouchterlony agar plates and by its ability to inhibit guanylate cyclase. Monoclonal antibodies against guanylate cyclase were produced by fusing the spleen cells of mice immunized with purified guanylate cyclase (only mice showing the presence of antibody against guanylate cyclase were used) with P 3X63Ag 8 myeloma cells using polyethylene glycol. The hybrid cells were isolated and grown in suspension culture. The hyrbid cells producing specific antibody against guanylate cyclase will be selected and cloned.

Major Findings: During the past year we have been able to purify large quantities of guanylate cyclase showing a single band in gel electrophoresis. The purified eznyme was able to convert 16 umole min^{-1} mg^{-1} GTP to cyclic GMP. The enzyme appears to contain 2 subunits of apparent M.W.59000. After repeated injections of purified enzyme in rabbits over a period of one year, the sera showed presence of specific antibody against guanylate cyclase. In mice, antibody appears to be produced within 2 months of injection. The antisera showed a very discrete single precepitin line against purified as well as partially purified gaunylate cyclase. It also inhibited guanylate cyclase. The production of monoclonal antibody is in progress.

Significance to Biomedical Research and Program of the Institute: Preparation of a specific antibody against guanylate cyclase by both conventional and monoclonal technique would pave the way for the production of antibodies against other guanylate cyclase as well as other enzyme systems. The availability of specific monoclonal and polyclonal antibodies will greatly help in the understanding of the role of cyclic GMP in physiological as well as pathological conditions.

Proposed Course of Project: We propose to characterize the antbodies prepared against guanylate cyclase and utilize them for elucidating the the enzyme guanylate cyclase in the retina and lung. The role of cyclic GMP in these tissues is not clearly understood and we hope that the learning of the localization of the enzyme may help in the understanding of the critical role of cyclic GMP in health and disease.

Publications:

Chader, G., Liu, Y., O'Brien, P., Fletcher, R., Krishna, G., Aguirre, G., Farber, D. and Lolley, R.: Cyclic GMP phosphodiesterase activator: Involvement in a hereditary retinal degeneration. Neurochemistry 1: 441-458, 1980.

Giri, S.N., and Krishna, G.A.: The effect of paraquat on guanylate cyclase activity in relation to morphological changes of guinea pig lung. Lung 157: 127-134, 1980.

Kapoor, C.L. and Krishna, G.: Noncompetitive inhibition of soluble guanyalte cyclase by 2'-deoxyguanosine-3'-monophosphate. Biochemical Pharmacology 28: 2861-2863, 1979.

Chader, G.J., Fletcher, R.T., Russel, P. and Krishna, G.: Differential control of protien kinase activities of the retinal photoreceptor. Cation effects on phosphorylation by adenosine and guanosine triphosphates. Biochemistry 19: 2634-2638, 1980.

Giri, S.N. and Krishna, G.: A simple and sensitive assay for prostaglandin synthesis in the guinea pig lung: Evidence for the formation of prostacyclin. In Methods in Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00846-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of Chemical Modification of Prostacyclicn on its Action

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Gopal Krishna	Chief, Section	LCP	NHLBI
OTHERS:	Nancy Kim	Chemist	LCP	NHLBI
	Mariam George	Vist. Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Effects of various chemical modifications of prostacyclin (PGI_2) on anti-aggregation of human platelets and on elevation of platelet cyclic AMP (cAMP) were investigated utilizing ^{3}H-adenine labeled human platelet-rich plasma. Elongation of the chain length of PGI_2 by one methyl group (20-methyl PGI_2) did not markedly alter the PGI_2 action on platelts while reducing this chain length by one methyl group (2-nor-metyl PGI_2) abolished the PGI_2 action on platelets in regards to both anti-aggregatory activity as well as increase in cAMP. Acetylenic analogs of PGI_2 were more active than PGI_2 and the increase of chain length by one methyl group (20 methyl 13,14 didehydr-PGI_2) markedly potentiated the activity against platelet aggregation as well on cAMP while introduction of a methyl group at carbon 16 markedly reduced both activities. Replacement of oxygen in the hetrocyclic ring of PGI_2 by a methylene group increased the chemical stability but markedly reduced the activity against aggregation as well on cAMP. Similar results were obtained with all analogs of PGI_2 when tested on platelet membrane adenylate cyclase.

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Project Description:

Objectives: Prostacyclicn (PGI_2) is a potent inhibitor of platelet aggregation. It presumably acts by increasing the synthesis of cAMP by activating platelet adenylate cyclase. Although PGI_2 plays an important physiological role in platelet functions, the study has been limited due to unstable nature of PGI_2 at physiologica pH. It is rapidly converted to 6-keto $PGF_{1\alpha}$, which has little effect on platlet aggregation. The main purpose of this study is to investigate the effects of various PGI_2 analogs on platelet aggregation and platelet cyclic AMP and to develop an analog of PGI_2 that is more potent and stable than PGI_2. The development of such an analog of PGI_2 would be useful not only in clinical practice but also in helping to elucidate the PGI_2 receptor interactions on human platelets.

Methods Employed: Human platelet righ plasma was incubated for 30 min with ^3H adenine (64 nM - 1 mC/ml). PGI_2 or its analogs was added to the blood platelets at various concentrations. The reaction was terminated with 5% TCA and the ^3H-cAMP that accumulated in response to the compounds was isolated by column chromatography on Dowex-50-H^+ followed by $Ba(OH)_2$ - $ZnSO_4$ precipitation. The recovery of cAMP was routinely monitored by addition of ^{14}C-cAMP. The results were compared with that obtained by radioimmunoassay method previously employed. Adenylate cyclase in the platelet membrane was measured as reported earlier.

Platelet aggregation was measured with a BIO-DATA platelet aggregometer. Dose-dependent inhibitory effects of PGI_2 and its analogs on ADP (50 uM) induced platelet aggregation were compared. Various analogs of PGI_2 were supplied by Dr. Grandolfi of Farmitalia - Carlo Erba of Milano, Italy.

Major Findings: Elongation of aliphatic chain in PGI_2 molecule by a methyl group (20 methyl PGI_2) did not alter the anti-aggregation action on platelet while decreasing the chain by one methyl group (20 nor-methyl PGI_2) almost abolished its capacity to increase cAMP level and to inhibit platelet aggregation.

In the acetylenic analogs as in 13,14-di-dehydro derivatives, having a methyl group of cabon-20 markedly increased the potency of the compound whereas a methyl group at carbon-16 decreased the inhibition of aggregation and cAMP elevation.

Carboprostacyclins which were PGI_2 analogs in which oxygen in the heterocyclic ring is replaced by methylene group had very little ativity and was comparable almost to that of 6-keto $PGF_{1\alpha}$, the hydrolysis product of PGI_2. Even though carboprostacyclins were more stable than PGI_2, they had very low activity towards the elevation of cAMP in platelets. Similar results were also obtained with all analogs of PGI_2 when tested against platelet membrane adenylate cyclase.

Significance to Biomedical Research and Program of the Institute: PGI_2 and its analogs are currently under intensive study for use in the treatment of a ide variety of disorders, including myocardial infraction, angina, vascuular occulsive diseases and hypertension. Synthesis of other PGI_2 analogs that retain the physiological function of the molecule, yet are stable when administered *in vivo* may be of critical importance in clinical practice.

Proposed Course of Project: In order to understand the exact physiological role and mechanism of PGI_2 platelet interaction, specific receptor binding studies will be carried out with various PGI_2 analogs and specific PGI_2 antagonists such as anacardic acids.

Publications: None

Awards: An award for Basic Research by Foundation of Fight for Sight.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00847-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Role of Calcium in CCl_4-induced Hepatotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Chenery	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI
OTHER:	None			

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Isolated hepatocytes maintained in culture have been used to study carbon tetrachloride (CCl_4) induced hepatotoxicity. Hepatocytes maintained in cultured for 1 day retain the capacity to metabolize aminopyrine and to activate CCl_4. Cell injury has been assessed by measuring leakage of cytoplasmic enzymes (e.g., lactic dehydrogenase: LDH), lifting of cells from the monolayer, morphological appearance and exclusion of trypan blue at different calcium concentrations and in the presence of the calcium ionophore A23187. No significant changes in covalent binding of CCl_4 are observed under these culture conditions. Release of LDH with ionophore (1 uM) or CCl_4 (1.5 mM) alone is decreased by complexing calcium with EGTA (5 uM). Injury due to ionophore and CCl_4, in the presence of high calcium, is approximately additive, and is characterized by extensive "blebing" of the plasma membrane when these agents are used in combination. The results reported here and by other laboratories implicate calcium as a modulator of cell injury by CCl_4.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Isolated hepatocytes in suspension have been used to study the metabolic activation and cytotoxicity of a number of drugs and xenobiotics. However, the ability to maintain these cells for extended periods of time is desirable in order to investigate events leading to cell death, subsequent to metabolic activation and covalent binding.

Hepatocytes isolated from male adult rats can be maintained in monolayer culture for considerable periods of time, whilst levels of drug-metabolizing enzymes are similar to those found in vivo. We have employed a monolayer technique to investigate the interrelationship between the role of calcium in CCl_4 hepatotoxicity, metabolic activation, and covalent binding of CCl_4.

Methods Employed: Isolated hepatocytes have been prepared by a two-step collagenase perfusion technique. Initially interstitial calcium is removed by the chelator EGTA, followed by perfusion with medium containing 5 mM calcium chloride and 0.05% collagenase for about 10 min. Isolated cells are then allowed to adhere to culture dishes for 1 hr before changing the culture medium. All plastic dishes are pretreated with poly-L-lysine.

Culture medium is saturated with carbon tetrachloride and then diluted to the appropriate concentration with the culture medium. In a typical experiment, monolayers are incubated with the appropriate media for a 2 hr period, before measurement of parameters of cell toxicity, such as enzyme release, cell detachment and cell morphology. Cell appearance is monitored by phase contrast, scanning and transmission electron microscopy.

The concentration of CCl_4 and its metabolism to chloroform is measured by gas chromatography equipped with an electron capture detector. Covalent binding of $^{14}[C]\ CCl_4$ to protein and lipid is measured by methods established in this Laboratory.

Major Findings: Isolated hepatocytes can be cultured in poly-L-lysine treated plastic culture flasks and petri dishes. Live cells attach to the surface within 1 hr, as long as sufficient serum is included in the culture medium. Few dead, if any, cells attach within this time. Optimum culture conditions require 10% horse serum, 0.02 i.u. insulin/ml and high concentrations of nicotinamide (10-25 uM) in the culture medium. Under these conditions cells flatten within 4-6 hr and form cord-like structures within 18-24 hr. These cords of cells are not seen in the absence of nicotinamide.

Cells cultured 18-24 hr retain 60-100% of drug-metabolizing capacity as judged by aminopyrine metabolism to 4-aminoantipyrine. This situation is found with hepatocytes from both control and phenobarbital pretreated animals. Hepatocytes from induced animals metabolize 5-6 fold more aminopyrine than do noninduced cells. Nicotinamide is not required to maintain high enzyme activity under these culture conditions.

Cells incubated in the presence of ^{14}C CCl_4 in the medium, covalently bind CCl_4 to protein and lipid. This binding is linear for at least 10 min and is proportional to substrate concentration in the range 1-3.5 mM. The extent of covalent binding is insensitive to the level of calcium in the culture medium and also to the ionophore A23187. Cells treated with CCl_4 detach from the monolayer and LDH leakage into the medium is observed. The extent of LDH leakage can be enhanced by raising the calcium concentration in the culture medium and by including the calcium ionophore A23187. In the presence of high calcium (3.6 mM) the ionophore alone causes approximately the same release of LDH as does 1.5 mM CCl_4 alone. The effect of CCl_4 and ionophore are additive, although CCl_4 alone at high concentrations (3.5 mM) can cause complete release of LDH within 2 hr. EGTA in the presence of low calcium particularly block the release of LDH by CCl_4. It thus appears that calcium may modulate cell death after injury due to CCl_4.

Significance to Biomedical Research and Program of the Institute: Many drugs cause liver damage which has been associated with metabolic activation and covalent binding. These results suggest that the damage caused by active metabolites of CCl_4 can be modulated by the availability of calcium.

Proposed Course of Project: To investigate the molecular mechanisms by which calcium modulates cell injury and the role of calmodulin in drug-induced hepatotoxicities.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 H1 00848-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Evidence by SEM for the Mediator Role of Calcium in Hepatotoxicity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mariam George	Vist. Fellow	LCP	NHLBI
	Richard Chenery	Vist. Fellow	LCP	NHLBI
	Gopal Krishna	Chief, Section	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Victor J. Ferrans, Pathology Branch, Ultrastructure Section, NHLBI

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug-Tissue Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Scanning electron microscopy (SEM) of liver cells treated with carbon tetrachloride (CCl_4) showed marked changes including formation of blebs on the surface of liver membrane which were markedly increased by addition of $CaCl_2$ and calcium ionophore, A 23187 and were markedly reduced by removal of calcium from the medium. The changes that occurred on the liver membranes in response to various treatments corresponded to various biochemical changes reported earlier.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: In another report we provided evidence, based on biochemical studies, for a definite role of calcium in drug-induced damage to the liver cells. One of the main objectives of this study has been to examine the changes that occur to liver cell membranes in response to hepatotoxicty by scanning electron microscopy and to examine whether calcium plays a role in these changes.

Methods Employed: The rat liver cells were prepared by collagenase perfusion of the liver and the cells were allowed to attach to plastic cover slips which had been coated with polylysine. The liver cells were treated for two hours with various concentrations of carbon tetrachloride in presence or absence of calcium and/or 10 µM calcium ionophore (A 23187). The cells were fixed with 2.5% glutaraldehyde at the end of various time incubations. The cells were normally fixed for 1-2 min and washed in 0.1 M cacodylate buffer pH 7.2. The cells were then fixed in 2% osmium oxide for 20-25 min and washed in the buffer and the cells were dehydrated through a series of alcohol concentrations. The cells were subjected to critical point drying and were coated with a thin layer of gold palladium and examined with a JEOL JSM 35 scanning electron microscope.

Major Findings: Under scanning electron microscopy the control cells appeared to have normal surface membrane with no evidence of any cell damage. When the cells were treated with carbon tetrachloride (0.5-2 mM) in the presence of calcium ionophore, A 23187 (10 µM), the cells exhibited various bizarre changes of the cell membrane including formation of numerous blebs. The cell damage to the membrane was greatly reduced when calcium was omitted from the medium. The changes that occurred to the cell membrane by CCl_4 were potentiated by addition of calcium and ionophore. These findings were similar to the biochemical changes reported earlier.

Signifiance to Biomedical Research and Program of the Institute: Scanning electron microscopy appears to be a reliable technique for examming surface changes that occur to the cell membrane in response to hepatotoxins. However, it is difficult to quantitate, but in combination with other biochemical parameters it would serve as a useful tool for studying the mechanism of toxicity induced by various drugs.

Proposed Course of Project: We propose to examine the liver cells grown in the culture flask rather than on the plastic cover slips and examine the changes that occur to the cell surface and the cell interior by combined use of phase, scanning and tansmission electron microscopy. We also propose to examine if calcium plays an important role in the toxicity produced by other compounds.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00854-03 LCP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanism of Hepatic and Renal Toxicity of Phenylbutazone

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Henry A. Sasame	Chemist	LCP	NHLBI
	Harriet M. Maling	Chief, Physiology Section	LCP	NHLBI
Others:	James R. Gillette	Chief, Lab. of Chemical Pharm.	LCP	NHLBI
	Wilford Saul	Chemist	LCP	NHLBI
	Kenneth Greene	Biol. Lab. Tech.	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Michael Boyd is in the Clinical Pharmacology Branch, NCI.

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.3	OTHER: 0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We previously reported that phenylbutazone caused liver necrosis in rats pretreated with both phenobarbital and diethylmaleate. However, studies carried out during the past year failed to reproduce these effects for reasons that are not clear. Experiments are now in progress to find a more sensitive species and conditions which can be correlated with a dose-related induction by phenylbutazone of hepatic damage.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The objectives of this project are to demonstrate the production of hepatic damage by large doses of phenylbutazone in a laboratory animal and to establish the relationships between covalent binding of phenylbutazone to liver microsomal protein and the production of liver damage in this species.

Methods Employed: Plasma concentrations of glutamic-pyruvic acid transaminase will be measured as indicators of possible hepatic damage. When a suitable species has been found and conditions have been established which are correlated with reproducible increases in plasma GPT after large doses of phenylbutazone, appropriate histologic slides of livers will be made and inspected for the extent and type of hepatic damage.

Major Findings: Hepatic damage induced by large doses of phenylbutazone is variable, both in otherwise untreated rats and in rats treated with phenylbutazone. Large doses of phenylbutazone did not produce renal necrosis in rats.

Significance to Biomedical Research and Program of the Institute: This study may give insight into the mechanisms responsible for the occasional cases of hepatic damage which have been resported in patients treated with phenylbutazone.

Proposed Course of Project: Experiments are now planned with other species, such as the hamster, guinea pig and mouse, in an attempt to find a species more sensitive than the rat to the toxic effects of phenulbutazone. When species and conditions are established which are reproducibly associated with hepatic damage, measurements will be made of covalent binding of phenylbutazone to liver microsomal protein, to explore the relationship between such binding and hepatotoxicity.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00856-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the renal reabsorption of cephalosporins in the rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Annie Arvidsson	Quest Worker	LCP	NHLBI
	Harriet M. Maling	Chief, Physiology Section	LCP	NHLBI
Other:	Wilford Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Physiology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.2	OTHER: 0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Renal clearance of cephaloridine was lower than inulin clearance at low plasma concentrations of cephaloridine and higher than inulin clearance at high plasma levels. Since changes in protein binding of the drug cannot account for the effects, the data suggest that cephaloridine is reabsorbed by the kidney in the rat, in addition to being filtered and secreted. In the anesthetized rat, cephaloridine increased significantly the rate of endogenous urate urinary excretion and the renal clearance of endogenous urate. The plasma concentration of endogenous urate was decreased. This uricosuric effect of cephaloridine suggests that uric acid and cephaloridine are reabsorbed by the same transport system. Experiments are now in progress to measure the effects of cephaloridine on the uptake of urate by rabbit renal cortical slices.

Project Description:

Objectives: In a study in healthy human volunteers, Arvidsson, Borga and Alvan (Clin. Pharmacol. Ther. 25: 870-876, 1979) reported that cephapirin and cephaloridine were reabsorbed in addition to being filtered and secreted. The objective of this project was to find an animal model for the reabsorption of cephalosporins and to determine the type of endogenous transport system which is involved. Transport systems under consideration included systems that reabsorb amino acids, urate and ascorbic acid.

Methods Employed: Rats are anesthetized with Chloropent Injection (Fort Dodge Labs., Inc.). The urinary bladder is catheterized with a polyethylene tubing inserted through the urethra. Blood samples are taken from a retro-orbital sinus at appropriate intervals.

Plasma and urine concentrations of cephalosporins are analyzed with a HPLC method. Amino acids are qualitatively determined in urine by a ninhydrin reagent. Urate is determined by a uricase method with a Sigma kit.

Major Findings: Cephapirin is rapidly and almost completely metabolized in the rat. Since the recovery of intact cephapirin in the urine was less than 1%, clearance measurements were not possible in the rat. In the hope of detecting reabsorption of cephapirin in a different species, cephapirin was infused intravenously in four rabbits to give a high (13 ug/ml) or low (3.4 ug/ml) plasma steady state concentration. No reabsorption of cephapirin or of deacetylcephapirin could be detected in the rabbit with clearance techniques.

Since there was no reabsorption in the rabbit, we looked for reabsorption of other cephalosporins in the rat. Clearance measurments with cephalexin did not indicate reabsorption. In contrast, the renal clearance of cephaloridine at plasma concentrations greater than 10 ug/ml was about 11 ml min^{-1} kg^{-1}. At lower plasma concentrations, the renal clearance dropped to about 3 ml min^{-1} kg^{-1}; this suggested renal reabsorption of cephaloridine in the rat. We therefore decided to use the clearance of cephaloridine in the rat as a model for the reabsorption of cephalosporins.

Cephaloridine (40 mg/kg in a bolus injection iv) increased the rate of endogenous urate urinary excretion in 9 rats from 1.3 ug min^{-1} to 2.3 ug min^{-1}. The renal clearance of urate rose from 0.25 ml kg^{-1} min^{-1} to 1.3 ml kg^{-1} min^{-1} and the plasma urate concentration fell more than 50%.

Experiments are now in progress to measure the effects of cephaloridine on urate uptake by rat renal cortical slices.

Significance to Biomedical Research and the Program of the Institute. If cephaloridine exerts an uricosuric effect in humans as in the rat, then cephaloridine should be given specific consideration as an antibiotic for treating an infection in a patient with gout.

Proposed Course of Project: A manuscript is now in preparation for publication.

Publications:

Maling, H.M., Saul, W., Yasaka, W.J. and Gillette, J.R. Effect of isoproterenol on the toxicity in rats of compounds eliminated by the kidneys. Pharmacology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00857-01 LCP

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Steady-state Pharmacokinetics of p-Aminohippurate (PAH) in Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Harriet M. Maling	Chief, Physiology Section	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharmacology	LCP	NHLBI
Other:	Wilford Saul	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Physiology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Drugs are frequently eliminated from the body by two or more organs, the relative contributions of which depends on the route of administration of the drugs. A study is in progress of the relationships between steady-state blood concentrations and urinary and biliary excretion of p-aminohippurate (PAH) during simultaneous infusion into the portal vein of PAH labeled with ^3H or ^{14}C and into the tail vein of PAH labeled with the other isotope. This study serves as a surrogate for a drug that is metabolized by the liver and excreted by the kidney.

Project Description

Objectives: One of us (JRG) has previously presented a pharmacokinetic analysis of the effects of the route of administration on the steady-state concentrations of foreign compounds and their metabolites in tissues when the substances are metabolized by the liver and excreted by the kidney (Ciba Foundation Symposium, in press). This analysis has produced the following simple equation:

$$\frac{Bp}{Bs} = 1 + \frac{Cl_R}{Q_H}$$

in which B_p is the steady-state rate of biliary excretion during continuous infusion into the portal vein and B_s is the steady-state rate of biliary excretion during infusion into a systemic vein, Cl_R is the renal blood clearance of the compound, and Q_H is the hepatic blood flow.

The objective of this study is to demonstrate the validity of this relationship in the rat and to calculate the hepatic blood flow during simultaneous intraportal and intravenous infusions of ^{3}H- or ^{14}C-PAH.

Methods Employed: Radiolabeled (^{14}C- or ^{3}H-) PAH was infused at a constant rate for two hours into the portal vein and PAH labeled with the other isotope was infused simultaneously at the same rate into the tail vein. By measuring the ^{3}H- and ^{14}C-radioactivity, the contribution of PAH infused by each route could be evaluated. A steady-state was assumed to exist when the rate of infusion was approximately equal to the sum of the renal excretion rate and the biliary excretion rate.

Major Findings: The ratio Bp/Bs was usually in the range 1.20-1.56. Hepatic blood flow varied from 40 to 80 ml kg $^{-1}$min^{-1} in different rats.

Significance to Biomedical Research and Program of the Institute: This study provides another method for calculating hepatic blood flow. It also tests the validity of the equation given in the Objectives and increases our understanding of the effects of different routes of administration of drugs.

Publications: Brown, E.A.B. and Maling, H.M. Effects of paraquat and related herbicides on the acetylcholinesterase of rat lung. Biochem. Pharmacol. 29: 465-466, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00917-05 LCP

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies on the Formation of Reactive Metabolites of Phenacetin and Acetaminophen

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Jack A. Hinson	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
	Terrence J. Monks	Vist. Fellow	LCP	NHLBI
	Mun Hong	Biol. Aid	LCP	NHLBI
	Kenneth Green	Biol. Lab. Worker	LCP	NHLBI
	James R. Gillette	Chief, Lab. of Chem. Pharm.	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.0	OTHER: 0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The hepatotoxicity of acetaminophen (A) is mediated by a reactive metabolite(s). We have previously shown that this metabolite is not formed by N-hydroxylation 3,4-epoxidation, or 2,3-epoxidation. In this work we show that that there is more than one reactive metabolite; more covalent binding occurs with ^{14}C-ring-A than with ^{14}C-A. Acetamide was found to be a microsomal metabolite of ^{14}C-acetyl-A. Acetamide formation plus covalent binding of ^{14}C-acetyl-A equaled ^{14}C-ring A binding. These data indicate two reactive metabolites of A. Ascorbic acid blocked acetamide formation and covalent binding of both labels. Addition of exogeneous protein to the microsomal incubation mixture increased covalent binding and decreased acetamide formation. These data are consistent with N-acetylimidoquinone being a acetreactive metabolite of A and hydrolysis of N-acetylimidoquinone yields acetamide plus benzoquinone, which also binds to protein. One electron reduction of N-acetylimidoquinone will yield N-acetylsemiquinone which may also be a reactive metabolite. The data are consistent with the concept that A is converted to N-acetylimidoquinone, by a previously unknown mechanism for cytochrome P-450.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Previous studies in our Laboratory have shown that the hepatotoxicity of acetaminophen is mediated by a reactive metabolite and that the hepatotoxicity of phenacetin is mediated by conversion to acetaminophen. The mechanism of formation and the structure of the reactive metabolite of acetaminophen, however, have remained elusive. The object of this project is to determine the structure of this metabolite and determine its mechanism of formation.

Methods Employed: Liver microsomes were isolated from hamsters by standard procedures. Acetamide was assayed by high pressure liquid chromatography. ^{14}C-Acetylacetaminophen was synthesized from p-aminophenol and ^{14}C-acetic anhydride by previously published techniques. All other methods were standard procedure.

Major Findings: More covalent binding was found using ^{14}C-ring-acetaminophen than with ^{14}C-acetyl-acetaminophen. Acetamide was determined to be a metabolite of ^{3}H-acetylacetaminophen; the ^{3}H-acetamide metabolite, which was isolated by HPLC from a microsomal incubation mixture, recrystallized with authentic unlabeled acetamide in the three different solvent systems. Acetamide formation plus covalent binding of ^{14}C-acetylacetamiophen equaled covalent binding of ^{14}C-ring acetaminophen. Ascorbic acid blocked binding of both acetaminophen labels. Addition of supernatant protein or boiled supernatant protein increased covalent binding and decreased acetamide. Glutathione decreased binding and did not alter acetamide formation. Glutathione plus supernatant, which contains glutathione transferases, decreased covalent binding, decreased acetamide and a glutathione conjugate was formed. In the presence of glutathione more reactive metabolite was accounted for as a glutathione conjugate than could be accounted for by covalent binding. More NADPH was oxidized in the absence of glutathione than in the presence of glutathione. These data are consistent with the view that N-acetylimidoquinone is a reactive metabolite of acetaminophen. Since hydrolysis of N-acetylimidoquinone would yield acetamide plus benzoquinone, benzoquinone may also be a reactive metabolite of acetaminophen. Since more NADPH is oxidized in the absence of glutathione than in the presence of glutathione apparently the reactive metabolite may be reduced to acetaminophen, whereas in the presence of glutathione it is conjugated. One electron reduction of N-acetylimidoquinone by NADPH cytochrome c reductase would yield N-acetylimidosemiquinone, which may also be a reactive metabolite. Thus there may be three reactive metabolites of Acetaminophen.

Significance to Biomedical Research and Program of the Institute: 1) The studies establish mechanisms for hepatotoxicity of acetaminophen and phenacetin. 2) Suggest a new mechanism for cytochrome p-450; oxidation of acetaminophen directly to N-acetylimidoquinone without the intermediatry of an oxygenated derivative. 3) Suggest a role of imidoquinone in toxicology. 4) Underscore complexities in identifying toxic intermediates.

Proposed Course of Project: Project will be terminated.

Publications:

Hinson, J.A., Andrews, L.S., and Gillette, J.R.: Kinetic evidence for multiple reactive metabolites of acetaminophen. Pharmacology 19: 237-248, 1979.

Hinson, J.A., Pohl, L.R. and Gillette, J.R.: A simple high pressure liquid chromatographic assay for the N-hydroxy derivatives of phenacetin, acetmainophen, 2-acetylaminofluorene, and other hydroxamic acids. Analytical Biochem. 101: 462-467, 1980.

Hinson, J.A.: Biochemical Toxicology of Acetaminophen. In Reviews in Biochemical Toxicology, vol. II, Hodgson, E., Bend, J. and Philpot, R. (eds.), Elsevier/North Holland, Inc. Amsterdam, 1980, pp. 103-129.

Hinson, J.A., Pohl, L.R., Monks, T.J., Gillette, J.R. and Guengerich, F.P.: 3-Hydroxyacetaminophen: A microsomal metabolite of acetaminophen. Evidence against an epoxide as the reactive metabolite of acetaminophen. Drug Metabolism and Disposition, in press.

Nelson, S.D., Vaishnav, Y., Gillette, J.R. and Hinson, J.A.: The use of ^{2}H and ^{18}O to examine arylating and alkylating pathways of phenacetin metabolism. Stable Isotopes: Proceedings of the Third International Conference, Academic Press, 1979, p. 385-392.

Pang, K.S., Taburet, A.M., Hinson, J.A. and Gillette, J.R.: High-performance liquid chromatographic assay for acetaminophen and phenacetin in the presence of their metabolites in biological fluids. Journal of Chromatography 174: 165-175, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00928-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Synthesis of Deuterated and Tritiated Derivatives of Enflurane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrence R. Burke, Jr.	Guest Worker	LCP	NHBLI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. Burke is a Research Associate in th Pharmacology-Toxicology Research Associate Program, NIGMS, Bethesda, Md. 20205

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Drug Enzyme Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Specifically deuterated derivatives of the inhalation anesthetic enflurane (CHF_2OCF_2CHClF) have been synthesized by a facile base catalyzed exchange. Tritiated enflurane has also been synthesized by this procedure. These labeled derivatives have been employed to determine the mechanism of metabolism of enflurane to the potential kidney toxin fluoride ion.

Project Description:

Objectives: To develop a facile and rapid method for the synthesis of deuterated and tritiated derivatives of the inhalation anesthetic enflurane.

Methods Employed: Dideuteroenflurane ($C^2HF_2OCF_2C^2HClF$) was prepared by heating to reflux for 2 hr a mixture containing enflurane, 1 M NaOD, and the phase transfer catalyst, Aliquat 336 (tricaprylmethylammonium chloride). The monodeuterodifluoromethoxy derivative of enflurane ($C^2HF_2OCF_2CHFCl$) was synthesized from dideuroenflurane by selective replacement of deuterium with hydrogen at the chlorofluoromethyl carbon. This reaction was conducted in the presence of H_2O, (pH 8-9) and Aliquat 336 at reflux for 2 hr. Monodeuterochlorofluoromethyl enflurane (CHF_2OCF_2CHClF) was synthesized from enflurane employing the later reaction conditions, in the presence of D_2O. Ditritiated labeled enflurane ($C^3HF_2OCF_2C^3HClF$) was synthesized by reacting enflurane in the presence of ^3H_2O, 1 M NaOH, and Aliquat 336 for 3 days at 67°C.

Major Findings: Employing the procedures outline above $C^2HF_2OCF_2$-C^2HClF, $C^2HF_2OCF_2CHClF$, $CHF_2OCF_2C^2HClF$, and $C^3HF_2OCF_2C^2HClF$ were prepared in yields of 50%, 66%, 33%, and 53%, respectively.

Significance to Biomedical Research and Program of the Institute: There have been recent reports indicating that enflurane anesthesia can produce renal changes in both man and rat. Since fluoride ion is believed to be responsible for this toxicity, it is important to be able to determine how enflurane is metabolized to fluoride ion. Based upon previous studies with related halogenated hydrocarbons, such as chloroform, halothane, and chloramphenicol, enflurane is likely metabolized to fluoride ion by an oxidative dehalogenation mechanism. However, this reaction may occur by initial oxidation of the C-H bond of the chlorofluoromethyl carbon or by oxidation of the C-H bond of the difluoromethyl carbon. One way to determine the relative importance of these pathways of metabolism is to compare the relative rates of defluornation of specifically deuterated derivatives of enflurane. Another approach is to determine the structure of the organic end products of metabolism. This can only be readily accomplished with a radiolabeled derivative of enflurane. Tritium labeled enflurane can be employed for these studies.

Proposed Course of Project: The project is terminated.

Publiations:

Burke, T.R., Jr. and Pohl, L.R.: Synthesis of deuterated and tritiated derivatives of enflurane. J. Labelled Compd., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00929-01 LCP

PERIOD COVERED
October 1, 1970 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanism Depletion Liver Glutathione by Hepatotoxic Halocarbons

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie L. Martin	Biologist	LCP	NHLBI
	Richard V. Branchflower	Chemist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI
	David Nunn	Biological Aid	LCP	NHLBI
	Terrence J. Monks	Vist. Fellow	LCP	NHLBI

COOPERATING UNITS (if any)

Robert Highet is in the Laboratory of Chemistry of the National Heart, Lung, and Blood Inst.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.4	OTHER: 0.3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

One hr after administration to phenobarbital (PB) pretreated rats the various halocarbons (3.73 mmole/kg i.p.) depleted hepatic glutathione (GSH) as follows: $CHCl_3$, 75%; $CBrCl_3$, 41%; $CHBr_3$ 14%; CCl_4 12%. All of these halocarbons were converted to GSCOSG when they were incubated for 10 min in air with rat liver microsomes from PB-pretreated rats and GSH. This derivative of GSH was also identified as a metabolite in bile in the living rat. Moreover, the relative amounts of formation of GSCOSG paralleled the GSH depleting activities of the various halocarbons. These results indicate that GSCOSG is formed by the oxidative metabolism of the trihalo (CHX_3) and tetrahalomethanes (CX_4) into potentially toxic carbonyl halides (COX_2) which are detoxified by their reaction with GSH to produce GSCOSG.

Project Description:

Objectives: To determine if the oxidative metabolism of trihalo and tetrahalomethanes to carbonyl halides (COX_2) is responsible for GSH depletion by these compounds.

Methods Employed: GSCOSG was synthesized from GSH and $^{14}COCl_2$ and was purified by preparative high pressure liquid chromatography (HPLC). Rats were pretreated with phenobarbital prior to the administration of $CHCl_3$, $CBrCl_3$, $CHBr_3$, and CCl_4 (3.73 mmole/kg, in sesame oil, i.p.). After 1 hr, liver GSH levels were measured by the method of Elman. The various halocarbons (5 mM) were also incubated with liver microsomes, a NADPH generating system and GSH (5mM). The formation of GSCOSG was determined by C-13 NMR and quantitated by HPLC. GSCOSG was also identified as a metabolite in bile in living rats of the various halocarbons.

Major Findings: One hour after administration to phenobarbital pretreated rats the halocarbons depleted hepatic GSH as follows: $CHCl_3$, 75%; $CBrCl_3$, 41%, $CHBr_3$, 14%; CCl_4, 12%. When incubated for 10 min with rat liver microsomes and GSH all of the compounds were converted to the same metabolite which was identified as the thiocarbonate ester of GSH (GSCOSG) by HPLC and C-13 NMR comparison with a synthetic standard. The percent conversions of each of the substrates to this product were as follows: $CHCl_3$, 7.2%; $CBrCl_3$, 2.3%; $CHBr_3$, 0.7%; CCl_4, 0.3%. The same basic pattern of formation of GSCOSG was also observed when this metabolite was measured in bile following the administration of these compounds.

Significance to Biomedical Research and Program of the Institute: These results explain how chloroform and other trihalo and tetrahalocarbons deplete liver GSH. They strongly indicate that these compounds are oxidatively dehalogenated into reactive and potentially toxic carbonyl halides. GSH protects the body from these compounds by rapidly reacting with them to form the thiocarbonate GSCOSG. The trihalocarbons (CHX_3) are likely metabolized by a direct oxidation to trihalomethanols (CX_3OH) which spontaneously dehydrohalogenate to carbonyl halides. The tetrahalocarbons (CX_4), however, may form the carbonyl halides by either initial reduction to form trihalocarbons (CHX_3) which are subsequently oxidatively dehalogenated to COX_2 or by direct oxidation of the C-X bonds to form trihalomethyl esters of hypohalous acids, CX_3OX. These compounds would be expected to react rapidly with tissue nucleophiles to produce COX_2.

Proposed Course of Project: We plan to determine if carbon-halogen bonds can be directly oxidatively dehalogenated. Understanding this process may be important in explaining how halogenated drugs such as halothane ($CF_3CHBrCl$) cause hepatotoxicity.

Pulications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00930-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Identification of an Organic Metabolite of Enflurane in Rat and Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrence R. Burke, Jr.	Guest Worker	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHER:	Richard V. Branchflower	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. David E. Lees is in the Department of Anesthesiology, Clinical Center, NIH. Dr. Burke is a Research Associate in the Pharmacology-Toxicology Research Associate Program, NIGMS, Bethesda, Md. 20205

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme-Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Difluoromethoxyldifluoroacetic acid ($CHF_2OCF_2CO_2H$) has been identified as a metabolite of enflurane (CHF_2OCF_2CHClF) in rat liver microsomes in vitro and in human urine by gas chromatography mass spectrometry. A radiochemical assay showed that this was the only organic metabolite of enflurane in the urine of these patients. The formation of the metabolite in rat liver microsomes requires NADPH and O_2, and is inhibited by SKF 525-A or $CO:O_2$ (8:2). When the C-H bonds of the CHClF group of enflurane or the CHCl group of isoflurane ($CHF_2OCHClCF_3$) are replaced with C-Cl bonds virtually no fluoride ion (F^-) is produced from either derivative in liver microsomes. These results indicate that cytochrome P-450 catalyzes the oxidative dehalogenation of CHF_2OCF_2CHClF at its CHClF group to form $CHF_2OCF_2CO_2H$, Cl^- and F^-. In contrast, the CHF_2 group does not appear to be appreciably susceptible to metabolic oxidative dehalogenation. Therefore, the CHF_2 group can be incorporated into the design of new inhalation anesthetics so that they will not be appreciably metabolized to the potential kidney toxin, F^-.

Project Description:

Objectives: The findings of a recent metabolic study in vitro with specifically deuterated derivatives of enflurane presented as a separate project report (Z01 HL 00934-01 LCP) suggests that enflurane is exclusively defluorinated by oxidation of the C-H bond of the CHClF group. The object of the present report was to test this idea by identifying the organic metabolite(s) of enflurane. Until now, F^- was the only identified metabolite of enflurane. In addition, the enzyme system catalyzing the formation of the organic metabolite(s) has been investigated.

Methods Employed: $[^3H]$ labeled enflurane was utilized for the incubations with rat liver microsomes. The $[^3H]$ labeled metabolites were purified and analyzed by high pressure liquid chromatography (HPLC). Final characterization was accomplished by electron ionization and isobutane chemical ionization gas chromatography mass spectrometry (GCMS). The metabolites of enflurane in the urine of one male patient following anesthesia were also analyzed and characterized by a combination of HPLC and GCMS. The enzyme system catalyzing the formation of the organic metabolite(s) of enflurane was investigated in rat liver microsomes by performing incubations under various conditions and with several derivatives of enflurane.

Aliquots of total urine of 3 patients which were collected for 15-24 hrs after anesthesia were tagged with $[^3H]$ labeled metabolite obtained by incubation of $[^3H]$ labeled enflurane with rat liver microsomes. The resulting urines were subjected to a derivatization procedure which utilized $[^{14}C]$ labeled ethanolamine to form a $[^{14}C]$ labeled amide derivative of the metabolite. The resulting derivatives were chromatographed using high pressure liquid chromatography (HPLC) and quantitated by liquid scintillation spectrometry. From the ratios of $[^3H]$ and $[^{14}C]$, the absolute amount of original urinary difluoromethoxydifluoroacetic acid was calculated.

Major Findings: Rat liver microsomes in the presence of NADPH and oxygen catalyzes the release of fluoride from enflurane and to a lesser extent from isoflurane. When C-H bonds of he CHClF group of enflurane and the CHCl group of isoflurane ($CHF_2OCHClCF_3$) were replaced with a C-Cl bond, virtually no F^- was produced from either derivative in liver microsomes. Difluoromethoxydifluoroacetic acid ($CHF_2OCF_2CO_2H$) was the only observed metabolite of enflurane (CHF_2OCF_2CHClF) in rat liver microsomes in vitro. Moreover, the formation of the metabolite in rat liver microsomes required NADPH and O_2, and was inhibited when SKF 525-A or $CO:O_2$ (8:2) were present in the reaction mixture.

A new analytical procedure for the quantitation of the major organic metabolite of enflurane, difluoromethoxydifluoroacetic acid, was also developed. Application of this technique in a study using human urine showed total urine levels of 90, 276 and 471 μmole of this meabolite in

15-24 hr urines of 3 patients following enflurane anesthesia. In addition, analysis of HPLC effluent by absorption at 210 nm showed that little, if any chlorofluoroacetic acid was present, indicating that little metabolism at the difluoromethoxy position had occurred.

Significance to Biomedical Research and Program of the Institute: The identification of $CHF_2OCF_2CO_2H$ as an organic acid metabolite of enflurane in rat liver microsomes in vitro and in human urine shows directly for the first time, that the C-H bond of the CHFCl group is a site of metabolic oxidation. Virtually no chlorofluoroacetic acid was observed, which would have been direct evidence of an oxidation of the C-H bond of the CHF_2 group. The apparent metabolic stability of the CHF_2 group is further indicated by the absence of F^- production from $CHF_2OCFC_2Cl_2F$ and $CHF_2OCCl_2CF_3$ where the only site of oxidative defluori- is the CHF_2 group.

The results of this experiment show that the CHF_2 can be incorporated into the design of new inhalation anesthetics without being appreciably metabolized to the potential kidney toxin, F^-.

Proposed Course of Project: We intend to study further the metabolism of enflurane and other general anesthetics in humans both in vivo and in vitro. The results of these studies should help to define more completely the mechanism(s) of metabolism of this group of drugs. This information can then be employed for the design of safer, more specific acting drugs.

Publications:

Gandolfi, A.J., White, R.D., Sipes, I.G., and Pohl, L.R.: Bioactivation and covalent binding of halothane in vitro: Studies with [^{3}H] and [^{14}C] halothane, J. Pharmacol. Exper. Therap., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00931-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Stereospecific Dealkylation of Oxyprenolol by Mixed-function Oxygenases

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Henry A. Sasame	Chemist	LCP	NHLBI
OTHERS:	Terrence R. Burke	Guest Worker	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI

COOPERATING UNITS (if any)
Dr. Terrence R. Burke is a Research Associate in the Pharmacology-Toxicology Res. Assoc. Program, NIGMS, Bethesda, Md. 20205.
Dr. Wendel Nelson, Univ. of Washington, Seattle, Washington.

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Inteaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.7	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

By virtue of having a center of assymetry at the C-2 carbon, the enantiometric difference in metabolism of oxyprenolol, a β-adrenergic blocking agent widely used in Europe, has been investigated. The metabolism of oxyprenolol by liver microsomes requires oxygen, TPNH and is inhibited by carbon monoxide atmosphere, piperonyl butoxide and SKF 525-A. Thus it appears to be catalyzed by cytochrome P-450. The comparative studies of the corresponding metabolism by two isomers, the S and R forms of oxyprenolol, by liver microsomes from rat, mouse, guinea pig, hamster and rabbit demonstrate clearly that the stereospecificity of the dealkylation and ring hydroxylation depends on animal species.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Preliminary work on the in vivo metabolism of oxyprenolol in rats carried out by Wendel Nelson et al. at the University of Washington revealed that more of the R isomer of oxyprenolol was converted to ring hydroxylated metabolites than was the S isomer. Their observation lead us to speculate that the difference in the metabolite(s) may be reflected upon the stereospecific metabolism of oxyprenolol by rat liver. Thus, a series of studes was undertaken to delineate the underlying mechanism of stereospecific metabolism of two isomers of oxyprenolol, that is S and R forms.

Methods Employed: In order to minimize the loss of acrolein (allyl aldehyde), a metabolite of oxyprenolol, the incubations were carried out in rubber stoppered Erlenmyer flasks. Acrolein formed from oxyprenolol during the incubathion was trapped by the presence of 30 mM semicarbazide. The reaction was terminated by the addition of two volumes of methanol to precipitate the microsomes. After removal of the protein precipitate, the aqueous methanol supernatant was evaporated to dryness and the metabolites of oxyprenolol were extacted into ethyl acetate from the aqueous phase (pH 9.15-9.20). Subsequently, the ethyl acetate phase was evaporated to dryness under a stream of nitrogen. The final residue was dissolved in aqueous methanol (1:1). The metabolites in the methanol were analyzed by high pressure liquid chromatography (Waters Associate), fitted with a preparatory, µBondapak TM/C_{18} column. Each metabolite eluted from the column was identified by GC-massspectrometry.

Major Findings: 1) The addition of oxyprenolol to liver microsomes isolated from rats pretreated with phenobarbital induced a typical type I spectral change, possessing a peak at 388 nm and a trough at 423 nm. 2) The N- and Odealkylation of oxyprenolol by rat liver microsomes from phenobarbital treated rats required oxygen and TPNH and were inhibited by carbon monoxide, piperonyl butoxide and SKF 525-A, suggesting that these metabolic pathways catalyzed by cytochrome P-450 dependent mixed-function oxygenases. 3) The profiles of the metabolites of oxyprenolol formed by liver microsomes from phenobarbital (PB) and β-naphthoflavone (BNF) treated rats revealed that both BNF and PB enhanced the rates of N- and O-dealkylation, but BNF pretreatment also enhanced ring hydroxylation of C-4 position. Interestingly enough, after PB pretreatment there was no detectable ring hydroxylation. 4) The relative rates of oxidation of the S and the R isomers of oxyprenolol depends not only on the animal species but also on the type of oxidation. For example, the rates of O-dealkylation of the S isomer by liver microsomes isolated from rats, rabbits and guinea pigs were greater than those of the R isomer, whereas with hamsters the reverse was observed. On the other hand, the rate of N-dealkylation of the R isomer by guinea pig was greater than that of the S isomer. No other species exhibited this stereospecific difference. Similarly the rates of ring hydroxylation at the C-4 positon of the R isomer by liver microsomes from rats, guinea pigs and rabbits were greater than those of the S isomer. Moreover, the rates of ring hydroxy- at the C-5 position of the R isomer by liver microsomes from mice and guinea pigs were greater than those of the S isomer. Interestingly enough hamster liver microsomes had a high specificity for O-dealkylation (2.5 times greater than that or rat or mouse but were devoid of any ring hydroxylase; they were thus similar to liver microsomes from rats treated with PB. 5) Kinetic analysis of oxyprenolol metabolism by liver microsomes from PB treated rats revealed two affinity sites for the N- and O-dealkylation of both isomers. The maximum velocity (V_{max}) of the high affinity site for O-dealkylation was roughly 50% greater than that of the R isomer, whereas, there was no difference in the V_{max} values between the two isomers for N-dealkylation. Conversely, the kinetic analysis of oxyprenolol metabolism by rat liver microsomes from BNF treated rats revealed that the V_{max} for the C-4 ring hydroxylation of the R isomer was twice that of the S isomer. There was no difference between the R and S forms for the O-dealkylation and between the R and S forms for the C-4 ring hydroxylation by liver microsomes from BNF treated rats. In addition, the K_m values for these two pathways are quite similar, which raises the possibility that the reactions are catalyzed by the same enzyme in BNF treated rats.

Significance to Biomedical Research and the Program of the Institute: A thorough understanding of the special arrangement of drug cytochrome P-450 interaction in hepatic microsomes through the use of stereoisomers should shed light on the mechanism of the cytochrome P-450 enzymes.

Proposed Course of Project: Since there are, at least, three distinct hydroxylations of oxyprenolol, namely, ring hydroxylation, N- and O-dealkylations, it is important to determine which reaction is catalyzed by which form of cytochrome P-450 in each species. The kinetic analysis of oxyprenolol metabolism from S and R isomers should be carried out with purified cytochroem P-450 enzymes isolated liver microsomes from either PB or BNF induced rats. In addition, immunochemical studies with antibodies against specific forms of cytochrome P-450 should provide a further understanding of oxyprenolol cytochrome P-450 interaction.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00932-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

New Metabolites of Chloramphenicol

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrence R. Burke, Jr.	Guest Worker	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHER:	Patricia Morris	Biologist	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Terrence R. Burke, Jr. is a Research Associate in the Pharmacology-Toxicology Research Associate Program, NIGMS, Bethesda, Md. 20205

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.4	0.4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The major pathway of chloramphenicol (CAP, $RNHCOCHCl_2$) metabolism in rat liver microsomes is oxidative dechlorination to CAP oxamic acid ($RNHCO_2H$). We have now, however, observed at least 5 additional minor metabolites of CAP by HPLC analysis of incubation mixtures. Two of these metabolites have been identified by comparison of their HPLC retention time and mass spectra with authentic compounds. One of the compounds is p-nitrobenzyl alcohol ($NO_2C_6H_4CH_2OH$). This metabolite is only produced under aerobic incubation conditions. The second compounds is monochloro-CAP ($RNHCOCH_2Cl$). In contrast to the first identified metabolite, this product is only produced under conditions of low oxygen tension. The mechanism of formation of these metabolites and their potential bone marrow toxicity is currently being evaluated.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To determine if a metabolite of CAP is responsible for aplastic anemia produced by this compound.

Methods Employed: Radiolabeled CAP was incubated with rat liver microsomes from phenobarbital pretreated rats and a NADPH generating system in an atmosphere of air or nitrogen. After 30 minutes, the reactions were stopped by precipitating the microsomal protein with methanol. The methanol supernatants were analyzed by HPLC and the metabolites were identified by mass spectroscopy.

Major Findings: When CAP was incubated with rat liver microsomes in air, 5 minor metabolites in addition to the major metabolite, CAP oxamic acid, were detected. One of the compounds has been purified by preparative HPLC and its HPLC retention time and mass spectrum correspond to an authentic sample of p-nitrobenzyl alcohol. When the incubations are conducted under anaerobic conditions, one major metabolite is observed. This compound has the same retention time and u.v. absorption spectrum as authentic monochloro-CAP.

Significance to Biomedical Research and Program of the Institute: Chloramphenicol is an important antibiotic. Unfortunately, approximately 1 in every 20,000 patients who are administered this drug develop aplastic anemia. How CAP produces this disease is unknown. We feel, however, that a minor metabolite of CAP may be responsible for the onset of aplastic anemia. Consequently, identification and testing of the metabolite described in this report may help elucidate this medical problem.

Proposed Course of Project: We plan to identify the remaining unidentified metabolites of CAP, to study the enzymatic mechanism for the formation, and to determine if they are toxic to bone marrow.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00933-01 LCP

PERIOD COVERED
October 1, 1979 to Sepember 30, 1980

TITLE OF PROJECT (80 characters or less)

Bone Marrow Toxicity of Chloramphenicol and its Metabolites

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Brion J. Gross	Costep	LCP	NHLBI
	Jackie L. Martin	Biologist	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.1	OTHER: 0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An assay has been developed for measuring bone marrow "growth" in primary tissue culture. This procedure involves isolating total bone marrow from rat femurs and measuring the incorporation of [^{3}H] thymidine into DNA and [^{14}C] glutamine into protein during 3 hours of incubation. The effect of CAP and its various identified and potential metabolites on these incorporations have been evaluated. At a concentration of 1 mM CAP inhibited bone marrow by 70%. Two metabolites of CAP were significantly more potent in inhibiting bone growth than CAP. These compounds are monochloramphenicol and p-nitrobenzaldehyde. Whether or not these metabolites or others are responsible for the bone marrow toxicity produced by CAP remains to be determined.

406

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: To develop an in vitro assay for determining if a metabolite of CAP could be responsible for the bone marrow toxicity produced by this drug.

Methods Employed: Bone marrow was obtained from femur of rats. Bone marrow growth was determined by measuring the incorporation of [^{3}H] thymidine into DNA and [^{14}C] glutamine into protein over a period of 3 hours of incubation. The relative bone toxicity of CAP and several of its known and potential metabolites was evaluated by determining their effects on DNA and protein synthesis.

Major Findings: The primary cultures of bone marrow cells incorporated [^{3}H] thymidine and [^{14}C] glutamine into DNA and protein, respectively during 3 hours of incubations. CAP at a concentration of 1 mM inhibited DNA and protein synthesis by approximately 70%. Of the various known and potential metabolites of CAP studied, monochloro-CAP and p-nitrobenzaldehyde showed the greatest inhibitory activity. At a 1 mM concentration monochloro-CAP inhibited DNA and protein synthesis by approximately 80%, whereas p-nitrobenzaldehyde produced a 98% inhibition.

Signifiance to Biomedical Research and Program of the Institute: Because aplastic anemia produced by CAP is rare, we have felt that a minor but exceedingly toxic metabolite may be responsible for this bone marrow disease. The assay reported in this investigation can be radily employed to test the relative bone marrow toxicity of metabolites of CAP. Two such compounds, monochloro-CAP and p-nitrobenzaldehyde appear to be more potent then CAP in inhibiting bone marrow. These compounds can be tested in vivo to determine if they produce aplastic anemia. If these or other metabolites of CAP are shown to be responsible for aplastic anemia produced by CAP, this information can be employed for the design of a safer derivative of CAP which is not metabolized to a bone marrow toxin.

Proposed Course of Project: Other newly identified metabolites of CAP will be tested for bone marrow toxicity in this in vitro assay. The procedure will also be adapted to study human bone cells in vitro. Moreover, we plan to determine if bone marrow cells can metabolize CAP into toxic metabolites.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 00934-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanism of Defluorination of Enflurane in Rat Liver Microsomes with Deuterated Derivatives

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Terrence R. Burke, Jr.	Guest Worker	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie L. Martin	Biologist	LCP	NHLBI
	John W. George	Chemist	LCP	NHLBI

COOPERATING UNITS (if any)

Dr. Burke is a Research Associate in the Pharmacology-Toxicology Research Associate Program, NIGMS, Bethesda, Md. 20205

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.4	0.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These results of this study indicate that the C-H bond of the difluoromethyl group of enflurane is not significantly susceptible to metabolic oxidative defluorination. This finding can be used for the rational design of new inhalation anesthetics which will not be appreciably metabolized to fluoride ion. The study also demonstrates an important use of specifically deuterated derivatives for readily determining the major pathways of defluorination of enflurane in various microsomal preparations.

Project Description:

Objectives: To employ specifically labeled deuterium derivatives of enflurane (CHF_2OCF_2CHClF) to determine whether this inhalation anesthetic is metabolized to the potential kidney toxin, F^- by oxidation of the C-H bonds of the difluoromethoxy group (CHF_2) or the chlorofluoromethyl group (CHClF).

Methods Employed: The rates of defluorination of enflurane (CHF_2-OCF_2CHClF) and its various deuterated derivatives in liver microsomes from rats pretreated with phenobarbital (PB) on isoniazid (ISO) were measured with a specific ion F^- electrode.

Major Findings: The results expressed as nmole F^-/mg protein/30 min for incubation with microsomes from PB or ISO pretreated rats are as follows: F^- released from CDF_2OCF_2CDClF (PB, 0.4; ISO, 2.5) and CHF_2OCF_2-$CDClF$ (PB, 0.4; ISO, 2.9) were nearly identical but significantly lower than that released from enflurane, CHF_2OCF_2CHClF (PB, 1.1; ISO, 12.3). In contrast, incubations with CDF_2OCF_2CHClF (PB, 1.0; ISO, 12.4) yielded virtually the same amount of F^- as found with enflurane (CHF_2OCF_2CHClF).

Significance to Biomedical Research and Program of the Institute: The results of these experiments confirm the general use of deuterium labeled compounds for the elucidation of pathways of bioactivation of various halocarbon drugs. The primary deuterium isotope effects observed in the present report strongly suggest that only the C-H bond of the CHClF group of enflurane (CHF_2OCF_2CHClF) is susceptible to oxidative defluorination. This finding can be employed for the design of a safer general anesthetic that is not metabolized to the potential kidney toxin F^-.

Proposed Course of Project: The general approach described in this project of employing deuterium labeled derivatives to elucidate metabolic and toxicologic mechanisms is being used in other projects.

Publications:

Burke, T.R, Jr., Martin, J.L., George, J.W. and Pohl, L.R.: Investigation of the mechanism of defluorination of enflurane in rat liver microsomes with specificially deuterated derivatives. Biochem. Pharm. 29: 1623-1626 (1980).

Sipes, I.G., Gondolfi, A.J., Pohl, L.R., Krishna, G. and Brown, B.R., Jr.: Comparison of the biotransformation and hepatotoxicity of halothane and deuterated halothane. J. Pharmacol. Exp. Therap., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00935 01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Induction of Enflurane Defluorination by Hydrazine Containing Compounds

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Branchflower	Chemist	LCP	NHLBI
	Lance R. Pohl	Sr. Staff Fellow	LCP	NHLBI
OTHERS:	Jackie Martin	Biolgist	LCP	NHLBI
	John George	Chemist	LCP	NHLBI
	Terrence R. Burke	Guest Worker	LCP	NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Chemical Pharmacology

SECTION
Enzyme Drug Interaction

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.5	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pretreatment of rats with isoniazid or hydrazine results in the increase rate of defluorination of the general anesthetic enflurane by rat liver cytochrome P-450. Other microsomal enzymes, such as epoxide hydrase, cytochrome b_5, and cytochrome P-450 reductase are not affected by this treatment. Analysis of the composition of rat liver microsomes by SDS polyacrylamide electrophoresis indicates that the pretreatment with isoniazid or hydrazine does not obviously alter the relative abundance of the various forms of cytochrome P-450. Moreover, addition of dilauryl phosphatidyl choline to microsomes treated with acetone and butanol (to remove lipid) restored the defluorination activity to the same activity as the controls. Thus it seems possible that pretreatment of rats with isoniazid may alter the lipid composition of the microsomal membranes and thereby change the conformation of the membrane bound cytochrome P-450 and its enzymatic activity.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: A recently reported case of F^- induced toxicity following enflurane administration is thought to have been associated with previous chronic administration of isoniazid. Indeed, we and other workers have demonstrated that pretratment of rats with isoniazid leads to increased *in vitro* defluorination of enflurane to F^-. The objective of the current investigation is to characterize further the biochemical nature of this inductive effect, to determine whether or not it is a generalized property of drugs and environmental pollutants containing the hydrazine moiety and to what extent this induction may affect the metabolism and/or toxicity of other drugs.

Methods Employed: [^{3}H] Enflurane was synthesized by an exchange reaction with an alkaline solution of tritiated water as illustrated in another report (Z01 HL 00928-01 LCP). Rat liver cytochrome P-450 catalytic activity was assayed by measuring the metabolism of enflurane to F^- and $CF_2HOCF_2CO_2H$. Cytochrome P-450 and cytochrome P-450 reductase, from the livers of control and treated animals, were partially purified via anion exchange chromatography. An HPLC assay for styrene oxide and styrene diol was developed and used to monitor epoxide hydrase activity. The assays used for cytochrome b_5, cytochrome P-450 reductase, glutathione transferase, glutathione and extraction of microsomal lipids were conducted by standard literature methods. SDS polyacrylamide gel electrophoresis was also performed by a standard literature procedure.

Major Findings: Pretreatment of Fischer and Sprague Dawley rats with isoniazod (50 mg/kg i.p. daily for 7 days) and Sprague Dawley rats with hydrazine (15 mg/kg i.p. daily for 7 days), or (50 mg/kg i.p. for 1 day) led to a marked increase of the *in vitro* defluorination of enflurane without changing the concentration or spectral properties of cytochrome P-450. This effect could not be produced by preincubation of control microsomes with isoniazid, hydrazine or N-acetylhydrazine, which suggests that it is not due to a simple activtion of an existing enzyme. Comparisons of the anion exchange column chromatography of the detergent solubilized microsomal preparation from control, isoniazid and hydrazine pretreated animals showed alterations in the second peak eluting from the columns of the isoniazid and hydrazine microsomes. However, SDS electrophoresis of the various microsomal preparations did not show significant differences in the relative abundance of their proteins.

Since hydrazine has been reported to cause fatty liver and alter the ratio of neutral lipids to phospholipid in the liver via induction of the activity of microsomal phosphatidate phosphohydrolase, we were led to believe that the increased defluorination activity might be due to changes in the microsomal membrane lipid composition.

In an attempt to more directly demonstrate the dependence or lack of dependence of enflurane defluorination on lipid, microsomal preparations from both one day and seven day pretreated animals were delipidated with butanol and acetone. These procedures resulted in a total loss of defluorinase activity which could be reconstituted to only control levels by addition of synthetic dilaurylphosphatidyl choline. These results, therefore, suggest that isoniazid and hydrazine might induce the defluorination of enflurane by altering the lipid composition of the microsomes.

In contrast to the increased activity of cytochrome P-450, pretreatment with isoniazid or hydrazine did not alter the activities of other microsomal enzymes such as cytochrome b_5, cytochrome P-450 reductase, or epoxide hydrase, nor did the treatments change the glutathione concentrations or GSH transferase activities in the cytosol.

Moreover, in contrast to the liver, the amount of enflurane defluorination by the kidney microsomes (5% of liver) was decreased by hydrazine pretreatment whereas there was no measureable defluorination by lung microsomes from either control or hydrazine pretreated animals.

Significance to Biomedical Research and Program of the Institute: These results indicate that the activation of enflurane defluorination by isoniazid could be produced by its metabolite hydrazine. At the present time, this effect does not appear to be due to an induction of new protein. Instead, it may be due to a change in the lipid composition of the microsomal membranes. This could potentially result in a conformational change of cytochrome P-450 which could alter its catalytic activity. Thus, isoniazid may increase the activities of cytochrome P-450 enzymes by a hitherto unsuspected mechanism.

Proposed Course of Project: The immediate direction of the research will focus on the question of whether the increase of enflurane defluorination following pretreatment with hydrazine is actually due to alterations of lipid and/or induction of a new form of P-450. Attempts will be made to determine whether liposomes from total lipids extracted from microsomes of either control or hydrazine pretreated animals can activate delipidated microsomes to differing degrees. These same total lipids will also be analyzed to determine whether or not their compositions are different. The individual bands from the anion exchange column of microsomes will be reconstituted and assayed to determine which, if any, band contains the defluorinase activity and whether or not this activity is affected by antibodies to known cytochrome P-450's. These bands will also be compared on SDS electrophoresis in search of a new band of cytochrome P-450. Rabbits, guinea pigs, mice and hamsters will be treated with hydrazine in search of a noninducible species or strain to use as a tool to probe the mechanism of induction. Further work will focus on the questions of whether or not other hydrazine containing drugs such as hydralazine, carbidopa, procarbazine and phenylbutazone have similar inductive effects.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00936-01 LCP

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Formation of Reactive Metabolites of Acetaminophen by Different Enzyme Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Henry A. Sasame	Chemist	LCP	NHLBI
	Jack A. Hinson	Sr. Staff Fellow	LCP	NHLBI
OTHER:	James R. Gillette	Chief	LCP	NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Chemical Pharmacology

SECTION

Enzyme Drug Interaction

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.3	0.3	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Acetaminophen may be converted to chemically reactive metabolites by either horseradish peroxidase and H_2O_2 or cumene hydroperoxide and hamster microsomes. Moreover, acetaminophen rapidly converts complex II of horseradish peroxidase to its native form indicating that acetaminophen can be oxidized by an one electron system. Whether the formation of the chemically reactive metabolite of acetaminophen formed by NADPH and hamster liver microsomes occurs by a peroxidase-like mechanism remains to be determined.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: It seems possible that N-acetylimidoquinone is the chemically reactive metabolite of acetaminophen formed by hamster liver microsomes. But the mechanism by which this metabolite might be formed is obscure. One possibility is that acetaminophen is oxidized by a peroxidase mechanism in which the substrate is oxidized by two single electron steps. Another possibility is that acetaminophen is oxidized by a single two electron step. To evaluate the plausibility of these mechanisms we have studied the oxidation of acetaminophen by horseradish peroxidase plus H_2O_2 and hamster liver microsomes plus cumene hydroperoxide.

Methods Employed: Liver microsomes were isolated from hamsters by standard procedures. Two unidentified metabolites were detected in high pressure liquid chromatography. Spectrometric determinations were carried out in Aminco DW-2 spectrophotometer.

Major Findings: On the addition of 2.5 nmoles of H_2O_2 to 2.5 nmoles horseradish peroxidase in the presence of 10 mg of bovine serum albumin, complex I (2 electron state) was formed instantaneously and then was rapidly converted to complex II (one electron state) owing to the presence of endogenous substrates of horseradish peroxidase in the preparations. On the addition of acetaminophen, however, the complex II was instantaneously converted to the native state of horseradish peroxidase. Thus, acetaminophen can be oxidized by a single electron mechanism. When 5, 2.5 or 1.25 nmoles of radiolabeled acetaminophen was added to the system, 1.0, 0.3 or 0.008 nmoles of the radiolabeled acetaminophen metabolite was covalently bound to the albumin. In the presence of 0.2 mM H_2O_2 and 1.0 mM acetaminophen, horseradish peroxidase converted acetaminophen to two unidentified metabolites, which were detected by high pressure liquid chromatography. The molar ratio of acetaminophen disappearance over H_2O_2 consumed was 2 to 1. In the presence of both H_2O_2 and bovine serum albumin ^{14}C-ring labeled acetaminophen was covalently bound to bovine serum albumin.

Cumene hydroperoxide added to hamster liver microsomes induced a spectral change having a trough at 418 mu and a peak at 440 mu, which indicates the formation of a complex thought to be analogous to complex I of horseradish peroxidase. As time elapses the magnitude of the trough becomes greater whereas the peak height at 440 mu slowly decreases and shifts toward higher wave length (445 mu). In the presence of acetaminophen the initial magnitudes of the trough and the peaks were much smaller but the peak at 440 mu increased with time. Consequently, there were two different points of inflection: with acetaminophen 402 mu and 435 mu; and without acetaminophen 432 mu and 450 mu. In the presence of 0.5 mM cumene hydrperoxide and 1.0 mM acetaminophen, 7.7 nmoles/ml of radiolabeled metabolite of acetaminophen was covalently bound to hamster microsomes.

When NADPH was added to hamster liver microsomes in the presence of acetaminophen, we could obtain no evidence for a spectral change comparable to the complexes I or II of horseradish peroxidase or the changes caused by cumene hydroxperoxide.

Significance to Biomedical Research and Program of the Institute: An understanding of the formation of reactive intermediate from acetaminophen should shed some light on the nature of its toxicity.

Proposed Course of Project: Hamster microsomal cytochrome P-450 enzyme will be solubilized and purified. Using the reconstituted compound P-450 enzyme systems, we shall compare the activation of acetaminophen by TPNH, H_2O_2 and of cumene hydroperoxide.

Publications: None.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTIONS ON CHEMICAL STRUCTURE AND STRUCTURAL NUCLEAR MAGNETIC RESONANCE
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1980, through September 30, 1981

The efforts of the Laboratory of Chemistry continue to be divided among two main activities, one chemical (Sections on Chemical Structure and Nuclear Magnetic Resonance) and the other biochemical (Section on Physiological Chemistry). The latter will be reviewed separately and appended to this report by its Section Head, Dr. J. Pisano.

In chemistry, our laboratory is currently active in three main areas: (1) isolation and analysis of biologically important compounds using gas and high pressure liquid chromatography, (2) structural analysis using mass spectrometry, nuclear magnetic resonance, X-ray single crystal methods, and (3) computer organization and analysis of scientific data.

Liquid chromatography continues to increase in application in the laboratory. It has been applied, both analytically and preparatively (H. Lloyd) to: 1) prostacyclin inhibitors (and potential molluscicides) from Anacardium occidentale (6-n-alkylsalicyclic acids); 2) identification of 2-pyrrolidone in rat brain; and 3) a sulfur-containing nucleoside from bacteria. The method is also being used for the analysis of plasma verapamil (T. Jaouni) where the technique has been improved to include analysis of the main metabolite, N-demethyl verapamil in the same analysis.

In mass spectrometry, the MS-9, even after reconditioning continues to be less than satisfactory, while the older LKB-9000 and newer LKB-2091 provide most analyses of the laboratory. A data system due to arrive shortly should make the latter even more valuable. Capillary GC systems were tried with only moderate success; improvements will have to be made on their injectors.

A chemical ionization modification kit has been received for the LKB-2091. Because the LKB-GC/MS company has gone out of business, we will be required to do all of our own service. This has been our custom for many years so we foresee no difficulty, but we have initiated a semiannual "LKB Newsletter" to maintain contact between users.

The $^{252}C_f$ plasma desorption mass spectrometer being built for us by Texas A&M has run its first spectra and should be completed on schedule. A recent visit disclosed that our earlier idea of using a minimal microprocessor-oriented data system is not practical. With DCRT support, a larger system will be investigated.

In nmr spectrometry, the Nicolet high field superconducting system has arrived and is now providing good routine spectra. In respect to its probe, we do not feel it is operating as well as it might; the company is attempting to overcome what may be certain inherent disadvantages of a wide range probe.

For the record, the collaboration of Dr. James Ferretti (DCRT) has been especially helpful during these early days of machine usage. Even at its present level, the apparatus has been valuable in determining the structures (R. J. Highet) of a series of dendrobated frog poisons of great interest in ion transport (J. Daly, NIAMDD).

Spin-lattice relaxation times of lipoproteins AI and AII have been conducted using ^{13}C enriched samples (E. Sokoloski). Difficulties with the buffer have been overcome and solute-solvent interactions of the proteins will be studied.

Lithium-7 and magnesium-25 resonances have been successfully observed with the new spectrometer and binding to ATP is under study.

In X-ray crystallography (J. Silverton) the apparatus continues to provide excellent data in spite of a temporary setback due to failure of a water cooling line. A special program has been written (J. Silverton) to allow measurement of weak reflections to any desired accuracy for determination of absolute configuration. Operation of the entire instrument has been eased by updating of the diffractometer computer last year. Complete structures have been obtained for epoxycolchicine (A. Brossi NIAMDD), palustric acid, an anti-malarial drug (J. Scovill, Walter Reed), very small crystals of a carcinogenic phenanthrene (D. Jerina NIAMDD) and polymorphs of the chiral and racemic tartaric acid methyl esters. A photochromic quinoline, reversibly turning red on exposure to UV was investigated, but X-rays caused the photochromism (due to impurities?) to cease so the problem was abandoned.

In an interesting twist, the structure of a synthetic nucleotide (T. Miles, NIAMDD) was refined to an R-factor of 2.3% and revealed unusual features that question accepted wisdom on conformation of these substances. The packing is not controlled by intramolecular hydrogen bonds and, as in the early Pauling-Corey DNA molecule, the phosphate groups are in the interior of the water-bridge-linked helices with the bases on the periphery. This result does not challenge the Watson-Crick model, but indicates that the earlier proposal was not as unreasonable as recently claimed.

The NIH/EPA Chemical Information System (G. Milne) now has 17 different components including mass spectral search, ^{13}C nmr search, nmr and crystallographic bibliographic search, structure and nomenclature search, crystal X-ray diffraction data search, powder diffraction data search, water pollutant search, search for chemicals in the Federal Registers, statistical analysis package, prediction of chemical properties from structure, regulatory status of chemicals, and spills of hazardous chemicals. Over 700 scientists use one or more of the systems and 35,000 searches are conducted monthly.

Other compounds whose structures have been solved in our laboratory this year include: the components of several secretions (pyrrolidones, pyrrolizidines, terpenes) from insects (M. Blum, U. of Georgia), 31-norcycloartenol, methyl-branched fatty acids from the anal gland of Herpestes sp.; a glutathione metabolite of chloroform (L. Pohl NHLBI); the structure of "diacetyl diethylmaleate"; and the analgesic ascorbic acid-glyoxal adduct (G. Fodor, U. of W.Va.).

In addition, a series of investigations are being conducted on urines of patients having unusual nerve disorders to see if there are lipid abnormalities of genetic origin (B. Blumenkopf, K. Engel,NINCDS), and lung chemotaxis factor (G. Hunninghake, R. Crystal NHLBI). These will be discussed next year.

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
SECTION ON PHYSIOLOGIC CHEMISTRY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1979 to September 30, 1980

Kallikrein-Kinin System: Kininogen has been localized in cells of distal tubules, collecting ducts, and medullary rays of human kidney by use of monospecific antibody raised to human plasma low molecular weight kininogen (LMW Kgn) and the indirect immunofluorescent technique. Fluorescence was usually more intense on the luminal side of the cells. No fluorescence was observed with antibody specific for high molecular weight kininogen (HMW Kgn). Normal urine also contains kininogen. Radioimmunoassays (RIA) for LMW Kgn and HMW Kgn have been used, together with a kinin RIA, to characterize urokininogen. All releasable kinin was associated with kininogen antigen. Human urokininogen antigen is a mixture of intact LMW Kgn and breakdown products of both HMW and LMW Kgns. Cells of the distal nephron probably are a source of LMW kininogen while HMW Kgn fragments represent filtered breakdown products of plasma HMW Kgn. Since kallikrein is also localized in the distal nephron, kinins are probably produced at this site through the interaction of local substrate and enzyme. The significance of numerous earlier studies from other laboratories involving injection of kinins into the renal artery may now be questioned as kinins are probably never present in the glomerulus in significant quantity and furthermore, they are rapidly destroyed in the proximal tubule but are recoverable in high yield from the distal tubule. The major site of kinin action in the kidney may be in the distal nephron. Other observations on kininogen: Immunoreactive LMW Kgn appears to be present in whole human saliva. HMW Kgn appears to be decreased in some patients with hereditary angioedema and carcinoid syndrome. Both HMW Kgn and prekallikrein appear to decrease in plasma of women immediately after parturition. In both human urine and parotid saliva, glandular kallikrein increases in normal volunteers on a low salt diet or after administration of Fludrocortisone. Immunoreactive glandular kallikrein has been detected in rat plasma using an RIA for rat urinary kallikrein (RUK). Gel filtration of rat plasma revealed 3 peaks of antigen. Only the third peak (Peak 3) showed immunological identity to RUK. Bilateral nephrectomy significantly increased the antigenic content of whole plasma antigen and of Peak 3. No significant difference in either whole plasma antigen or gel filtration profile was seen when pancreatectomized rats were compared to sham-operated controls, but both whole plasma antigen and the antigenic content of Peak 3 were significantly reduced after bilateral submandibular/sublingual gland excision. Antigen was detected in rat renal lymph but the level was not significantly different from plasma levels and no arteriovenous difference in antigen could be detected across the kidney. Peak 3 was indistinguishable from purified rat glandular kallikreins by gel filtration. Purified Peak 3, however, had no activity against kininogen or the chromogenic substrate S-2266, was not activated by trypsin, and showed a consistently smaller M_r (29 500) on SDS-polyacrylamide gel electrophoresis than that of RUK (32 000). Hence, glandular kallikrein circulates in rat plasma in an inactive form. The submandibular and sublingual glands appear to be the major source of the plasma antigen and the kidney plays an important role in the clearance and/or metabolism of glandular kallikrein from plasma.

Two highly specific and potent polypeptide inhibitors of Hageman factor (Factor XII) have been purified from corn kernels (M_r = 3 200-4 200; pI = 8.3). These inhibitors have very similar K_is, 2.4 and 3.3 x 10^{-8} M, respectively; form 1:1 molar complexes with trypsin and Hageman factor fragments; have arginine at the P_1 position of the reactive site; and cause marked prolongation of the activated partial thromboplastin time of human plasma. While both inhibit trypsin, they show no or negligible inhibiton of human plasma and urinary kallikreins, plasmin, α-thrombin, hog pancreatic kallikrein, bovine Factor Xa, and α-chymotrypsin. Such narrow-spectrum serine proteinase inhibitors are extremely rare and thus may be useful in biochemical and clinical studies involving Hageman factor. Affinity chromatography of both inhibitors on trypsin-agarose causes a considerable loss of inhibitory activity towards Hageman factor. No loss of activity was seen if chromatography was performed on anhydrotrypsin-agarose. An identical pattern of results was seen with the lily bulb inhibitor of human urinary kallikrein. SDS-PAGE of human plasma prekallikrein activated by brief contact with catalytic amounts of Hageman factor fragments showed a heavy chain of M_r 53 000 and two light chains of M_r 40 000 and 37 000, corresponding to the 88 000- and 85 000-dalton forms of prekallikrein observed by us and others. Further incubation in the presence of excess Hageman factor corn inhibitor converts the 53 000-dalton chain into two fragments of M_r 33 000 and 20 000. This further degradation is presumably caused by plasma kallikrein itself.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01002-07 CH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Nuclear Magnetic Resonance of Natural Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: E. A. Sokoloski	Chemist	CH NHLBI

COOPERATING UNITS (if any)
J. Osborne, MD IR NHLBI
J. Costa, M CN N

LAB/BRANCH
Laboratory of Chemistry

SECTION
Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Carbon-13 nuclear magnetic resonance of human apo-proteins AI and AII is being used to examine the interactions between the proteins. Proton nmr of frozen protein solutions give residual signals from water molecules excluded from the ice lattice because of solvent-solute interactions.

Observation of lithium and magnesium complexes by nmr are in early stages.

PHS-6040
(Rev. 10-76)

Objectives: The research is concentrated in two main areas 1) studies of lipoproteins A-I and A-II complexes, and 2) observation of less familiar ions like magnesium and lithium in the presence of complexing agents found in biological systems. During the past year we have been preparing the foundation for future work with these systems.

Carbon-13 studies of the human apo-protein A-II were started over a year ago with several attempts at spin-lattice relaxation time (T_1) determinations of the A-II which had been modified by reaction with carbon-13 enriched iodo acetic acid for the carbon-13 studies. Spin-lattice relaxation times of solutions of this material were measured; unfortunately, we were misled by a resonance from the buffer which occurred at the position expected for the enriched protein carbon resonance. Annoyingly, this signal responded to changes in parameters just as we had expected the protein itself to. Nevertheless, we were able to show an unexpectedly strong solvent-solute interaction which will be useful in future studies. Subsequent experiments with a different buffer system have provided data with a spin-lattice relaxation value of 0.43 seconds for A II. We are now collecting data on the variation of this value for mixed AI-AII solutions. As an addition to the above experiments, we have examined the solvent interactions by observing the proton resonances of the solvent in the crystalline state. Resonance lines from solids are not ordinarily observable, giving extremely broad lines. Any residual resonance can be attributed to solvent which is either bound in the hydration shell of the protein or otherwise interacting with the protein and excluding it from the ice lattice. Observations of several buffer systems, lipoproteins and chymotrypsinogen give such resonances. Using this method, we propose examining the solvent-solute interactions of the apo-protein AI and AII.

The second category of work in progress is the study of metal ions with our newly acquired NTC-360 multi-nuclear spectrometer. Both lithium-7 and magnesium-25 resonances have been observed during the past two months. Preliminary concentration vs. chemical shift and half-bandwidth measurements of lithium chloride solutions have been completed. The next step will be to observe complexes of the lithium with compounds of interest like adenosine triphosphate to determine variations of chemical shift with binding. Spectra of magnesium-25 in the presence of ATP and EDTA showed half-bandwidths of 140 HZ ± 10HZ. The larger quadrupole of this nucleus will make it more difficult to observe complexes at concentrations available in blood. We expect that some improvements in our spectrometer will still allow useful applications of this nuclei in our studies of complex formation in protein systems.

Publications: none

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01003-08 CH

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure of Natural Products Using Instrumental Methods

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H.M. Fales	Chief, Lab. of Chem.	CH NHLBI
OTHER:	T. Jaouni	Chemist	CH NHLBI

COOPERATING UNITS (if any)

none

LAB/BRANCH
Laboratory of Chemistry

SECTION

Chemical Structure Section

INSTITUTE AND LOCATION

NHLBI, NIH Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The HPLC analysis of verapamil in plasma has been modified to include its metabolite, demethylverapamil.

The structures of a pyrrolizidine in S. fugax has been elucidated and synthesized. The structures of gastrolactone and diethyldiacetylmaleate have been revised.

Urines of patients with unusual neuropathies have been examined for lipid abnormalities.

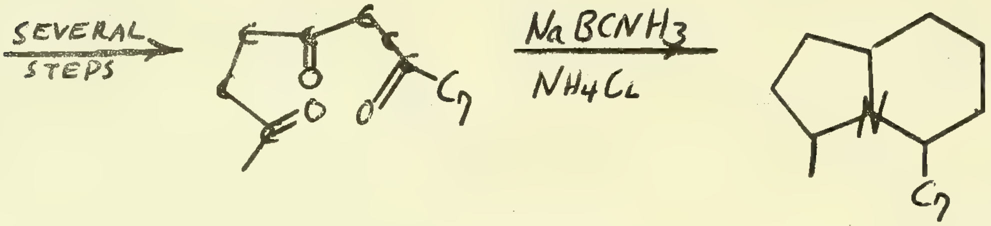
Lung chemotactic factor has been found not to be a prostaglandin.

Analgesic ascorbic acid-glyoxal conjugates have been examined by GC/MS.

The $^{252}C_f$ mass spectrometer being constructed at Texas A & M is operational.

1) Work has continued on the analysis of verapamil (M. Leon and D. Rosing, Cardiology Branch, NHLBI). Improved HPLC columns have allowed the separation and determination of the major metabolite, Ndemethylverapamil. Absence of the metabolite has been correlated with unusual sensitivity in some patients. The method is now in routine use and a paper describing it has been published.

2) With M. Blum (Dept. of Entomology, Univ. of GA) the identity of new constituents (1) of a "thief ant" (S. fugax) implicated as a disease vector in hospitals, has been elucidated and synthesized as shown.

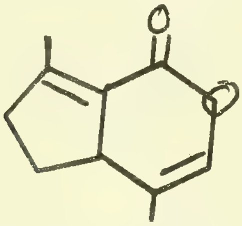


1

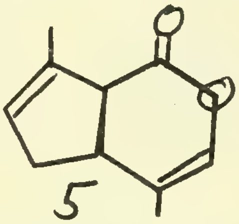
The structures of all possible stereoisomers of 1 have been elucidated using ^{13}C and ^{1}H nmr and correlated with the natural product.

A general method for the synthesis of 1,4 diketones and their conversion to 2,5-dialkylpyrrolidines, the toxins of certain fire ants, has been developed starting with the available related aldehydes and unsaturated ketones.

The structure of gastrolactone (4), a defensive constituent of Gastrophysa cyanea, has been synthesized and found not to be identical with the natural product whose structure has been revised to 5. Synthesis of 5 is in progress.

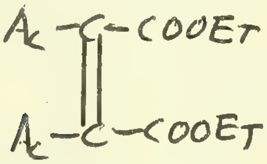


4

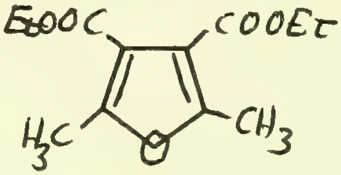


5

3) The structure of diethyl diacetylmaleate (2), a valuable potential synthon reported by another group, has been corrected using ^{13}C nmr and ms. The product is the furan (3). Attempts are currently underway to prepare 2 by a different route.



2



3

4) With B. Blumenkopf and K. Engel (NINCDS), the urines of a series of patients exhibiting unusual neuropathy have been investigated using GC/MS. Excesses of dicarboxylic acids have easily identified in the urines of several suggesting the possibility of genetic lipid defects.

5) With G. Hunninghake and R. Crystal, the nature of the lung chemotaxis factor is being investigated. Using quantitative GC/MS it has been shown that the active component cannot be one of the prostaglandins. Alkylacetylglycerylphosphoryl cholines are a likely possibility, and these are being studied.

6) With G. Fodor (Univ. of W. Va.) ascorbic acid--glyoxal conjugates having unusual morphinelike properties have been investigated using GC/MS. Earlier proposed structures have been discarded in favor of simple hemacetals. The reason for their analgesic action is difficult to comprehend.

7) The MS9 spectrometer has been nearly completely rebuilt and should be in service soon. The $^{252}C_f$ mass spectrometer being constructed at Texas A ± M is operational but requires adjustment and additional added devices (servomotors, reflectron, variable detector, etc.).

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2. Lundgren, D. W. and Fales, H. M. Metabolism of putrescine to 5-hydroxy-2-pyrrolidone via 2-pyrrolidone. J. Biol. Chem. 10: 4481-4486, 1980.
3. Norden, B., Batra, S. W. T., Fales, H. F., Hefetz, A. and Shaw, G. J. Anthrophora bees: Unusual glycerides from maternal dufour's glands serve as larval food and cell lining. Science 207: 1095-1097, 1980.
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5. Fales, H. M. and Highet, R. J. On the reported preparation of diethyl diacetylmaleate. J. Org. Chem, 45: 1699-1700, 1980.
6. Jones, T. H., Blum, M. S. and Fales, H. M. Chrysomelidial by chromyl chloride oxidation: A revised structure for gastrolactone. Tetrahedron Lett. 21: 1701-1704, 1980.
7. Jones, T. H., Franko, J. B., Blum, M. S., and Fales, H. M. Unsymmetrical 2,5-dialkylpyrrolidines via reduction amination of 1,4-diketones. Tetrahedron Lett. 21: 789-792, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01004-09 CH

PERIOD COVERED
October 1, 1979 - September 30, 1979

TITLE OF PROJECT (80 characters or less)

Characterization of Natural Products

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H.A. Lloyd	Research Chemist	CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this work is to determine the structures of physiologically active compounds isolated from plant or animal tissue. High pressure liquid chromatography is used to purify the new compounds for nmr and mass spectrometry study.

428

PHS-6040
(Rev. 10-76)

1. Cashew nutshell extract (in collaboration with G. Krishna, CP, NHLBI): A successful analytical and preparative separation of the components of "anacardic acid" extracted from "Anacardium occidentale" was achieved by high pressure liquid chromatography. "Anacardic acid" is a mixture of 6-n-salicylic acids: C_{13} to C_{17} side-chain homologues with 0, 1, 2 or 3 double bonds. The various compounds were collected individually and analyzed by derivatization and mass spectrometry. Pharmacological and toxicological testing have shown their role as specific inhibitors of prostacyclin receptor in human blood platelets and of the C_{15} triene as a potential molluscicide against B. glabata which harbors schistosomes.

2. (In collaboration with Drs. G. Kapadia and Y. Shukla, Howard University.) Examination of various plants extracts by GC-MS and identification of pure compounds of pharmacological interest was continued. An unusual compound from a Smilax species was identified by mass spectrometry and n.m.r.: 31-norcycloartanol, a triterpene alcohol possessing a 9:19 cyclopropane ring.

3. Insect pheromones (with Dr. M. Blum. U. of Georgia). In a continuing study of the myrmecocystus ants and termites several new species were examined. A number of mono and sesquiterpenes and esters of long-chain acids and alcohols were revealed by GC-MS. Other insect secretions (with Dr. A. Hefetz, Tel Aviv University) from sceliphron, tapinoma, catalyphus, etc. were also studied.

4. (with A. Hefetz, Tel Aviv U.) Anal gland secretions of Herpestes sp. (mongoose) were examined. GC-MS after derivatization revealed that they consisted of a complex mixture of mono, di, tri and tetra methyl-substituted fatty acids. With one exception all the acids are 4-Me branched; a large amount of a 2-Me substituted acid was found in the male Herpestes secretion only.

5. A considerable amount of time was spent in an effort to isolate a bacteria-produced, unknown, sulfur-containing nucleoside (with Dr. M. Lipsett, BM NIAMDD) for structure determination by mass spectrometry. The nucleoside and the free base were successfully isolated by high pressure liquid chromatography but were too unstable for further characterization.

6. (with Dr. D. Lundgren, PM NIAMDD). An HPLC method for the identification and quantitation of 2-pyrrolidone isolated from the brain of rats was developed.

Publications:

1. Kapadia G., Shukla Y., and Lloyd H.A. Revised structure of paederside, a novel monoterpene S-methyl thiocarbonate Tetrahedron Lett. 1937-1938 (1979).

2. Gomez-Sauchez C.E., Holland O.B., Murphy B.A., Lloyd H.A., and Milewich L. 19-Nor-Deoxycorticosterone: A Potent Mineralcocorticoid Isolated from the Urine of Rats with Regenerating Adrenals. Endocrinology 105 708-711 (1979).

3. Lloyd H.A., Denny C., and Krishna G. A Simple Liquid Chromatographic Method for Analysis and Isolation of the Unsaturated Components of Anacardic Acid. J. Liquid Chromatog. Accepted for publication (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL 01005-09 CH

PERIOD COVERED October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
X-ray Structural R & D for Physiologically Important Molecules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.V. Silverton	Research Chemist	CH NHLBI
OTHERS:	J. Scovill	Walter Reed Army Medical Center	

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Chemical Structure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Crystallographic studies where the method provides unique or most rapid solutions to structural problems. Development and use of Direct Methods for large ordered molecules.

430

PHS-6040
(Rev. 10-76)

a) Peptides and Nucleotides: The peptide aspect of this part of the work has not preceeded as rapidly as had been hoped because of difficulty in preparation of single crystals.

The synthetic nucleotide, 2-amino8-methyl-adenosine-5'-monophosphate, has been studied in detail and the experimental results have yielded the most accurate X-ray structure of a nucleotide or nucleoside ever reported. The results indicate the accuracy attainable by careful experimental technique and modern methods. The R-factor is 2.3% (almost the theoretical limit) and the accuracy, despite the presence of the phosphorus atom, is better than many light atom structures. Many aspects are of importance in nucleotide studies particularly for understanding the conformation of polynucleotides an, in several respects, the "accepted wisdom" in nucleotides may need re-evaluation. Some arguments as to the bonding and bond lengths in phosphate groups will have to be revised, the molecular conformation is unusual, being syn-C3'-endo (again unexpected), and the packing of the molecules does not involve intramolecular hydrogen bonds but is almost totally controlled by water bridges and local charge balance (the molecule is a zwitterion). Considerable work has been carried out to attempt to understand the reasons for the observations and the energetics of the situation.

The molecular packing is very unusual and consists of a set of three crystallographic helices linked by water bridges with the phosphate groups in the interior and the bases on the periphery. While the present work is undoubtably a special case and does not by any means vitiate the Watson-Crick nucleic acid model, the results bear an intriguing resemblance to the early triple stranded Pauling-Corey model for the nucleic acids.

b) Chemical Problems of Sterochemistry and Structure:

1. "Epoxycolchicine". The spectroscopic properties of this compound led to doubts about the assigned epoxide structure but could not give a unique answer. While the use of the latest techniques was necessary to solve the problem, the crystal structure shows that the compound is not an epoxide, but is 10,11-oxy-10,12a-cyclo-10,11-seco-11,12-dihydroxolchicine. The crystallographic results are in full accord with the chemical work.

2. Palustric Acid. The conformation of this terpenoid compound, while generally accepted, was not known with certainty or in full detail. Analysis of the conformations found by X-ray crystallography (the structure is fully refined at the present time) should prove useful in sesquiterpenoid chemistry.

3. (E,Z,E)-[4-(3,4-dihydro-2,6-dimethyl-3-(methylthio)-1,2,4-triazin-5(2H)-ylidene-2-butenylidene] methylhydrazinecarbodithioate. (with Captain J. Scovill, Walter Reed Army Medical Center).

This compound, besides holding this year's record for the longest name, is a synthetic product of interest for its anti-malarial activity. The structure could not be determined with certainty by chemical methods but has been fully elucidated by X-ray techniques.

4. 2,8-di(trifluoromethyl)-6-methyliminocyclohexyl-quinoline. This is again a compound where chemical proof of structure and sterochemistry was inadequate. The crystals possess the intriguing property of reversible turning bright red on irradiation with ultra-violet light and it originally seemed that accurate electron density work might be useful. The structure was solved but, after X-ray irradiation, the photochromism ceased. Because of this change, it does not seem useful to study the problem further. In addition, one of the trifluoromethyl groups unexpectedly shows disorder and thus accurate electron density work might be presently impossible. Mass spectroscopic investigation by Mr. W. Comstock of our laboratory appears to indicate that the crystals contain very small quantities of isomeric impurities which might actually be the source of the photochromism.

5. Possible isomers of 9-bromo-10-acetyl-hexahydrophenanthrene. The importance of this compound lies in the investigation of carcogenicity and chemical methods were inadequate to distinguish among several possible isomers. The compound also represented a technical challenge since the crystals were very small indeed and the structural problem would not have been attempted a few years ago. There was another problem in that the compound shows radiation damage. Despite difficulties, an entirely adequate answer has been obtained and confidence in working with very small crystals has been increased.

c) Optical Activity and Polymorphism in Dimethyl Tartrate (with Dr. H.M. Fales). The detection of optical purity by physical methods is of interest to this laboratory. Dimethyl tartrate is a simple compound showing optical activity but, despite the fact that it has been much investigated from about 1900 onwards, the literature shows many conflicting reports. There are apparently three known "polymorphs" of the optically active species, all with different melting points and crystal habits and two forms of the racemate. Seeding melts of racemate or optically pure forms with the different crystals produces different results. Only the meso compound has a published crystal structure and shows one crystal form.

The confusion in the literature now appears to be partially due to the effects of humidity and ambient temperature. Two of the optically active forms have been successfully obtained and elucidated crystallographically. We intend to attempt to obtain the third form which, although probably possessing the lowest energy state, is difficult to prepare. The crystal structure of the stable form of the racemate has been determined but, so far, we have been unable to obtain the metastable form as usable single crystals.

The explanation of the polymorphism currently appears to involve both conformation and hydrogen bonding.

d) Miscellaneous Topics. There have been several chemical problems this year which have been solved without full crystal structure determination by semiautomatic determination of space group and cell dimensions- a by no means trivial advantage of possessing a modern automatic diffractometer.

A program for the diffractometer to allow automatic measurement of weak X-ray reflections to any reasonable desired accuracy has been written. The program should prove useful in determining absolute configurations. Programming was made much easier by last year's updating of the diffractometer computer system.

Publications:

1. Silverton, J.V. The structure of colchiceine ethyl acetate-water solvate. Acta Cryst. B35, 2800-03, 1979.

2. Silverton, J.V., Ziffer, M. and Ziffer, H. Structure and steriochemistry of condensation products from 1-morpholino-1-cycloheptene and methyl vinyl ketone. J. Org. Chem 44: 3959-5961, 1979.

3. Cantrell, T.S. and Silverton, J.V. Chemistry of 1,2:5,6-dibenzocyclooctatetraene dianion. J. Org. Chem. 44: 4477-4481, 1979.

4. Mauger, A.B., Stuart, O.A., Highet, R.J., and Silverton, J.V.: Synthesis and conformation of cyclo(THR-D-VAL-PRO-SAR-MeALA). in Gross, E and Meienhofer, J (eds.) Peptides. Structures and Biological Function. Rockford, Ill, Pierce Chem. Co., 1979, pp. 237-240.

5. Brossi, A., Rosner, M., Silverton, J.V., Iorio, M.A., and Hufford, C.D. Revision of the structure of 'epoxycolchicine'. Helv. Chim. Acta 63, 406-412, 1980.

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7. Bright, W.M., Cannon, J.F., Langs, D.A., and Silverton, J.V. 1,7,7-Trimethyl-bicyclo[2.2.1]hepta-2,3-dione, $C_{10}H_{14}O_2$. Cryst. Struc. Comm, 8, 251, 1980.

8. Scovill, J.P., and Silverton, J.V. An unusually facile ring opening reaction in the pyridine system. J. Org. Chem., 45, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01006-09 CH

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Characterization of Natural Materials

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R.J. Highet	Chief, Structural NMR Section	CH NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Chemistry

SECTION
Structural Nuclear Magnetic Resonance Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

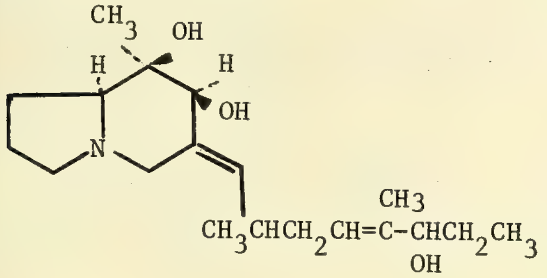
☐ (a1) MINORS ☐ (a2) INTERVIEWS

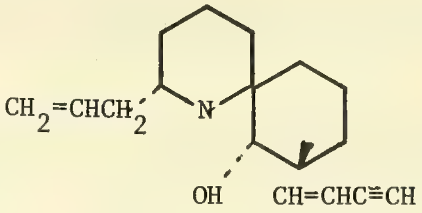
SUMMARY OF WORK (200 words or less - underline keywords)

Dendrobatid Alkaloids. Two alkaloids of the Pumiliotoxin A series and one of the histrionicotoxin series have been assigned structures based on nmr and mass spectral comparisons with alkaloids of known structure.

Metabolism of drugs. The product of microsomal transformation of chloroform has been shown to be the dithiocarbonate of glutathione.

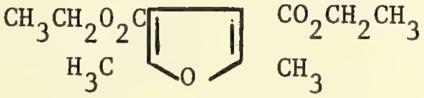
1. Dendrobatid Alkaloids: Studies with Dr. John W. Daly of NIAMDD have elucidated the structures of three alkaloids from the neotropical poison frog Dendrobates pumilio. Allopumiliotoxin B1 corresponds to I,



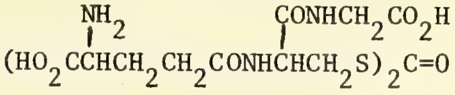


and Allopumiliotoxin B2 is the C-7 epimer of II. The minor alkaloid 259 has been shown to be II.

2. Transformations of Diethyl Diacetylsuccinate: Collaborative investigations with H.M. Fales (LC) have shown the reaction product of diethyl diacetylsuccinate with sulfuric acid to be the furan III, rather than diethyl diacetylmaleate as reported.



3. Metabolism of Chloroform: In collaboration with L. Pohl (NHLBI, CP), the product of the metabolism of chloroform by liver microsomes has been shown to be the glutathione dithioester IV.



$$(\mathrm{HO_2C\overset{NH_2}{\overset{|}{C}}HCH_2CH_2CONH\overset{CONHCH_2CO_2H}{\overset{|}{C}}HCH_2S})_2\mathrm{C{=}O}$$

Publications:

1. Mauger, A.B., Stuart, O.A., Highet, R.J., and Silverton, J.V. Synthesis and conformation of cyclo (Thr-D-Val-Pro-Sar-MeAla). in Gross, E. and Meienhofer, J. (eds.) Peptides, Structures and Biological Function. Rockford, IL, Pierce Chem. Co., 1979, pp 237-240.

2. Daly, J.W., Tokuyama, T., Fujiwara, T., Highet, R.J., Karle, I.L. A new class of indolizidine alkaloids from the poison frog, Dendrobates tricolor. X-ray analysis of 8-hydroxy-8-methyl-6-(2'-methylhexylidine)-1-azabicyclo [4.3.0]nonane. J. Amer. Chem. Soc. 102, 830 (1980).

3. Fales, H.M., and Highet, R.J. On "Diethyl diacetylmaleate". J. Org. Chem. 45: 1699-1700, 1980.

4. Highet, R.J., Covey, D.F., and Robinson, C.H. Carbon-13 NMR spectra of 5,10-secosteroids; a transannular intramolecular hydrogen bond. J. Org. Chem. In press.

5. Highet, R.J., Burke, T.R., Trager, W.F., Pohl, L., Menard, R.H., Taburet, A.M. and Gillette, J.R. Carbon-13 nuclear magnetic resonance studies of spironolactone and several related steroids. Steroids 35, 119-132, 1980.

6. Highet, R.J., "The Characterization of Dendrobatid Alkaloids" Robert Rowan, III Memorial Symposium, p. 24, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01008-10 CH

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Use of Digitial Computing in Problems in Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.W.A. Milne	Research Chemist	LC	NHLBI
OTHER:	C.L. Fisk	Staff Fellow	LC	NHLBI
	S.R. Heller	Computer Specialist	MIDSD	EPA
	R.J. Potenzone	Chemist	MIDSD	EPA

COOPERATING UNITS (if any)
EPA, NBS, FDA, NIOSH, NSF, Agencies in U.K., Switzerland, Finland, Japan, and Hungary

LAB/BRANCH
Laboratory of Chemistry

SECTION
NHLBI-NIH, Bethesda, MD 20205

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The NIH-EPA Chemical Information System, comprising 17 different components such as spectral data and bibliographic data bases, is under systematic development in a project that involves the collaborative efforts of 41 different groups in the U.S., Europe and Japan.

Components of this system include a mass spectral search system, a carbon-13 nmr spectral search system, nmr and crystallography bibliographic search systems, a structure search program, searchable data bases of x-ray diffraction data for crystals and for powders, a searchable data base of water pollutant data and a data base of chemical compounds cited in the Federal Register.

The entire system is being delivered to members of the international scientific community via networked computers in the private sector. All who use the system do so on a fee-for-service basis.

The NIH-EPA Chemical Information System (CIS) is currently being supported by seven agencies of the U.S. Government and involves the active collaboration of 41 different groups in 13 different countries.

Components of the CIS that are now operating in the private sector include the mass spectral search system, the carbon-13 nmr search system, an nmr spectral bibliographic search system, a statistical analysis package, a program for prediction of chemical properties, a search system for three different types of x-ray diffraction data and a structure and nomenclature search system. There are, in addition, data bases of water pollution data, data pertaining to the regulatory status of specific chemicals and a data base pertaining to responses to spills of hazardous chemicals. A total of over 700 scientists in North America, Europe and Australasia use one or more of these systems and more than 35,000 separate searches are conducted per month.

Several other components are currently being developed as a joint project between NIH/EPA and NBS. These include a data base of toxicities of consumer products, a file of thermodynamic properties and an infra red spectral search system.

Publications:

1. Meisel, W.S., Jolley, M., Heller, S.R., and Milne, G.W.A.: The Role of Pattern Recognition in the Computer-Aided Classification of Mass Spectra, Anal. Chim. Acta, 112, 407-416 (1979).

2. Hopfinger, A.J., Potenzone, R., Pearlstein, R., Kikuchi, O., Shapiro, M., Milne, G.W.A., and Heller, S.R.: Structure-Activity Analysis in the Classification of Toxic Chemicals, Ch. 20 (pp. 385-409) in Proc. Symp. on Safe Handling of Chemical Carcinogens, Vol. 2, Ann Arbor Press, Ann Arbor, MI.

3. Heller, S.R. and Milne, G.W.A.: The NIH-EPA Chemical Information System, In Chemcorner, Database, 2, 69-79 (1979).

4. Fisk, C.L., Milne, G.W.A., and Heller, S.R.: The Status of Infrared Data Bases, J. Chrom. Sci., 17, 441-444 (1979).

5. Heller, S.R., and Milne, G.W.A.: The NIH-EPA Chemical Information System in Support of Structure Elucidation. Vest. Slovensk. Kem. Drust., 26, 37-42 (1979).

6. Milne, G.W.A., and Heller, S.R.: The NIH-EPA Chemical Information System. In "Information Policy for the 1980's", pp. 85-120. Learned Information, Oxford, U.K. (1979).

7. Marquart, R.G., Marquart, L., Mintz, S.A., McGill, J.R., McDaniel, J., Heller, S.R., and Milne, G.W.A.: The NIH-EPA CIS Federal Register Search System. Online, 4, 45-49, (1980).

8. Heller, S.R., and Milne, G.W.A.: Online Spectroscopic Data Bases. Amer. Lab., 12, 33-48, (1980).

9. Milne, G.W.A., Heller, S.R., Heller, R.S., and Martinsen, D.P.: The NIH-EPA Chemical Information System. Adv. Mass Spec., 8B, 1578-1581, (1980).

10. Heller, S.R., and Milne, G.W.A.: The NIH-EPA Chemical Information System in Support of Structure Elucidation. Anal. Chim. Acta, 122, 117-138, (1980).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01012-07 CH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Peptide Biochemistry

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	J.J. Pisano	Head, Sec. of Physiol. Chem.	CH NHLBI
OTHER:	M. Bledsoe	Research Chemist	CH NHLBI
	P. Highet	Research Chemist	CH NHLBI
	H. Yoshida	Visiting Associate	CH NHLBI

COOPERATING UNITS (if any)
J. Gardner, Chief, Digestive Diseases Branch, DD NIAMDD
J. Gallin, Chief, Bacterial Diseases Section, LCI NIAID

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.7	1.1

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Peptide Biochemistry: Extracts of venom sacs from yellow jackets, hornets and wasps, as well as Gila monster venom contain peptides which have profound effects on human neutrophils and guinea-pig pancreatic acinar cells. The extracts inhibit enzyme release associated with neutrophil activation, they inhibit chemotaxis, and they can cause enzyme release. Extracts also cause the release of amylase from guinea-pig pancreatic acinar cells by mechanisms involving mobilization of cellular calcium and an increase in cellular cyclic AMP. The extracts are being fractionated by high performance liquid chromatography (HPLC) using a newly available column which gives separations superior to any we have obtained previously using other columns. A simple technique has been developed for rapidly desalting peptide solutions. To facilitate intra-laboratory reproducibility, a two-step gradient system has been developed as an alternative to our previously reported gradient system for the rapid analysis of amino acid phenylthiohydantoins. Several peptides have been coupled to chicken serum albumin and used to produce antibodies in burros and rabbits. Among the antibodies obtained it has been possible, for the first time, to produce antibodies against the amino-termini of bradykinin and angiotensin III.

PHS-6040
(Rev. 10-76)

Objectives:

1. Produce monospecific antibodies against the amino and carboxyl ends of bradykinin, angiotensin I, angiotensin II, angiotensin III, bombesin, ranatensin, and mastoporan and develop RIA's for these peptides.

2. Develop a rapid and reproducible stepwise elution method for the separation of amino acid phenylthiohydantoins by HPLC.

3. Evaluate and/or develop various methods for the separation of peptides by HPLC.

4. Isolate new biologically active peptides from insect venom, animal venom and amphibian skin.

Major Findings: Antigen Synthesis and Antibody Production

1. N-tert-butyloxycarbonyl-bradykinin was synthesized and the carboxyl terminus coupled to chicken serum albumin by the active ester method using 1-hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Four moles of bradykinin were coupled per mole of chicken serum albumin. Burros were immunized initially with 2 mg of antigen and given boosts of 1-3 mg monthly for 6 months.

2. Bradykinin was coupled to chicken serum albumin by the active ester method using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Six moles of bradykinin were coupled per mole of chicken serum albumin.

Burros were immunized initially with 2 mg of antigen and given boosts of 1-3 mg monthly for 6 months. Rabbits were immunized initially with 1 mg and given boosts of 1 mg monthly for 6 months. Animals were bled one week following injection.

3. N-tert-butyloxycarbonyl-angiotensin III was synthesized and coupled to chicken serum albumin by the active ester method using 1-hydroxybenzotriazole and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. Twenty-six moles of angiotensin III were coupled per mole of chicken serum albumin.

Rabbits were immunized initially with 1 mg of antigen and given boosts of 1 mg monthly for six months.

4. Mastoporan was coupled to chicken serum albumin by the active ester method using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide. The reaction was carried out at pH 5.0 in order to minimize coupling at the three lysine residues of mastoporan. Four moles of mastoporan were coupled per mole of chicken serum albumin.

Rabbits were immunized initially with 1 mg of antigen and given boosts of 1 mg monthly for three months. The animals were bled one week following injection.

5. The following peptides were coupled to chicken serum albumin by the active ester method using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide:

Ranatensin (27 moles coupled per mole CSA)
Bombesin (48 moles coupled per mole CSA)
Angiotensin I (22 moles coupled per mole CSA)
Angiotensin II (32 moles coupled per mole CSA)
Mastoporan (4 moles coupled per mole CSA)

Rabbits were immunized initially with 1 mg of antigen and given boosts of 1 mg monthly for 3 months. The animals were bled one week following injection.

Evaluation of New Antibodies for RIAs

The progress of antibody formation by burros and rabbits to peptides coupled to chicken serum albumin was monitored, and radioimmunoassay conditions were established for the most promising.

RIAs for Bradykinin

Serum from one burro immunized with bradykinin with the N-terminal end free can be used for RIA at a dilution of 1:4000 over a range of 8-1000 pg of standard or sample. ED_{50} (the amount required to displace 50% of tracer) is 93 pg. Polisteskinin, des-arg-1 bradykinin, lysyl-bradykinin, and methionyl-lysyl-bradykinin do not cross-react over a range of doses equivalent to 50-5000 pg of bradykinin. Cross-reactivity with two samples of kininogen was 0% and 4%--about equivalent to the probable level of free kinin contaminating the samples. Hence this antibody might be useful for detecting bradykinin in plasma, where interference of kininogen is a problem.

Serum from one rabbit with antibodies directed against the C-terminus can be used at a dilution of 1:163,000 over a range of 8-1000 pg. ED_{50} is 39 pg. Cross-reactives are: polisteskinin, 30%, des-arg-1 bradykinin, 9%, lysyl-bradykinin, 114%, methionyl-lysyl-bradykinin, 135%, tyrosine-8-bradykinin, 85%, kininogen, 2-15%.

One more burro and 3 rabbits treated with N-terminal-free bradykinin produced no antibodies. Two more rabbits and 2 burros immunized with C-terminal-free bradykinin produced poorer antibodies than the one described.

RIAs for Angiotensin II and III

Iodine-125 derivatives of angiotensin II and III were prepared with specific activities of 244 and 350 Ci/ g, respectively (15% and 20% incorporation). Sera of rabbits immunized with N-terminal-free angiotensin III and carboxy-terminal-free angiotensin II were tested.

Serum from one rabbit immunized with A III, used at a dilution of 1:25, 600, could detect A III over a range of 4-2000 pg with less than 1% cross-reactivity from A II.

Serum from another rabbit immunized with A III, at a dilution of 1:34,000, with ^{125}I A II as the tracer could detect A II and A III about equally over a range of 4-2000 pg, with ED_{50} of 122 pg for A II, 141 for A III.

The remaining rabbits produced poorer antibodies.

Bombesin, Mastoporan, and Ranatensin.

Attempts to evaluate these antibodies have been delayed by the problem of binding to surfaces which these peptides present.

Analysis of Amino Acid Phenylthiohydantoins by HPLC

In order to improve the reproducibility and decrease the cost of the analysis of amino acid phenylthiohydantoins by HPLC, we have developed a system for the stepwise elution and separation of the PTH-amino acids using a .46 x 15 cm Dupont ODS column.

In an attempt to optimize the separation of the PTH-amino acids, the effects of varying a number of different parameters, such as flow rate, pH, and acetate concentration were investigated.

Increasing the flow rate improved the resolution of PTH-proline and PTH-tryptophan but only at the expense of PTH-lysine and PTH-phenylalanine. Resolution of PTH-serine and PTH-glutamine was also improved by increasing the flow rate. Changing the flow rate by as little as 0.1 ml/min. resulted in significant differences in resolution.

Changes in the pH of the acetate buffer resulted in dramatic shifts in the positions of the basic PTH-amino acids, PTH-histidine and PTH-arginine, while the positions of the acidic PTH-amino acids, aspartic acid and PTH-glutamic acid, were relatively unaltered.

Changes in the acetate concentration of the buffer also had a pronounced effect on the retention of PTH-histidine and PTH-arginine. An increase in the acetate concentration of the buffer resulted in decreased retention of both PTH-histidine and PTH-arginine. This suggests that the basic PTH-amino acids may participate in an ion-exchange type of interaction with surface silanol groups rather than a simple ion-pair type of mechanism.

Changes in the acetate concentration of the buffer also had a significant effect on the resolution of PTH-serine and PTH-glutamine. Resolution of PTH-serine and PTH-glutamine was troublesome and required an acetate concentration of at least 0.03 M sodium acetate.

Knowledge of the effects of such parameters as flow rate, pH, and acetate concentration on the separation of the PTH-amino acids can be used to compensate for experimental and column variation. With a few minor modifications of our system, separation of all of the PTH-amino acids should be possible in less than thirteen minutes.

Analysis of Peptides by HPLC

The usefulness of the following HPLC systems for the separation of peptides was investigated:

Column	Solvent System
Dupont CN	TEAP, CH_3CN
Biorad ODS	0.1 M $NaClO_4$, CH_3CN
Dupont TMS	0.1 M $NaClO_4$, CH_3CN containing 0.005M sodium phosphate

While all three systems proved to be useful for the separation of peptides, peak shapes were superior with the latter system. Moreover, using this system, peptides such as mellitin, LBK, and substance P, which often stick to reversed phase columns or elute only with significant tailing, were eluted as fairly sharp peaks with little or no tailing. This system has been used in our laboratory for the analysis of venom sac extracts with good results.

Isolation of New Biologically Active Peptides

In the search for new biologically active peptides, we have examined venoms from the following:

1. Vespula pensylvanica (yellow jacket)
2. Vespula germanica (yellow jacket)
3. Vespula maculifrons (yellow jacket)
4. Polistes annularis (wasp)
5. Dolichovespula maculata (hornet)
6. Vespa crabro (European hornet)
7. Gila monster
8. Tityus serrulatus (scorpion)
9. Naja melanoleuca (black cobra)

Insect venom sacs were extracted with both 80% methanol and 1% acetic acid and the extracts compared by bioassay, HPLC, and gross weight. The biological activities of the methanolic and acetic acid extracts were comparable. The acetic acid extracts, however, contained a larger quantity of high molecular weight material. Extraction with 80% methanol, therefore, appeared to be superior for the isolation of small molecular weight substances.

The methanolic extracts of each of the venoms listed above were examined by HPLC using a system developed in our laboratory. An aliquot of the methanolic extract from Vespula pensylvanica was chromatographed by HPLC. Fractions corresponding to the major peaks were collected and aliquots hydrolyzed for amino acid analysis. Amino acid analysis revealed the presence of two peptides with amino acid compositions identical to those of vespulakinins 1 and 2. In addition, amino acid analysis of a third peak revealed the presence of an additional peptide, containing 22 amino acid residues. This peptide may be a new peptide of biological interest. The amino acid composition of this peptide is shown in the following table.

Amino Acid Composition of Peptide from Vespula Pensylvanica (residues/mol)

ASP	2.24
THR	0.98
SER	3.20
GLU	3.40
GLY	4.90
ALA	2.08
VAL	0.88
ILE	1.08
LEU	2.11
LYS	1.23
ARG	1.11

Since the HPLC system which we have employed contains non-volatile buffers, it was necessary to develop a method for desalting the column fractions before submitting them for bioassay. The use of small ODS-silica cartridges (Sep-Pak) for this purpose was investigated and found to be a rapid method for desalting peptide fractions with good recoveries.

The crude methanolic extracts were being screened for the following types of biological activity:

1. platelet aggregation
2. mast cell degranulation
3. rat uterus contracting activity
4. histamine release from human basophils and rat peritoneal mast cells
5. inhibition of oaubain binding to microsomal $Na^+ - K^+$ ATPase
6. inhibition of F-Met-Leu-Phe-induced chemotaxis and inhibition of enzyme release and stimulation of enzyme release
7. amylase secretion from pancreatic acinar cells

None of the crude extracts exhibited any histamine releasing activity from human basophils or oaubain binding inhibition. The following crude extracts, however, did stimulate amylase secretion from pancreatic acinar cells:

Vespula pensylvanica
Vespula germanica
Vespula maculifrons
Vespa crabro
Tityus serrulatus (scorpion)
Naja melanoleuca (black cobra)
Gila monster

The extracts vary in their spectrum of activities. They inhibit enzyme release associated with neutrophil activation, inhibit chemotaxis and cause enzyme release. Extracts also show variable but potent activity in the release of amylase from acinar cells. The mechanism of release involves mobilization of cellular calcium and increase in cellular cyclic AMP.

Proposed Course

Efforts will be directed toward the isolation and characterization of biologically active peptides from natural sources such as venoms and amphibian skin.

Publications:

Kokas, E., Pisano, J., Crepps, B.: Villikinin: Characterization and Function. in "Comprehensive Endocrinology" series editor L. Martinin. Gastrointestinal Hormones Edited by G.B.J. Glass, p. 899-910. Raven Press, New York, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01016-10 CH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical Biochemistry of the Kallikrein-Kinin System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.J. Pisano	Head, Section on Physiological Chemistry	CH NHLBI
OTHER:	P.L. Herring	Chemist	CH NHLBI
	P.F. Highet	Chemist	CH NHLBI
	M. Perkins	Chemist	CH NHLBI
	J.V. Pierce	Research Chemist	CH NHLBI
	D. Proud	Visiting Fellow	CH NHLBI
	K.N. Yates	Chemist	CH NHLBI
	D. Horwitz	Senior Investigator	HE NHLBI
	H. Keiser	Deputy Chief	HE NHLBI

COOPERATING UNITS (if any) B. Alving, Bureau of Biologics, FDA, Bethesda, Maryland
J. Niebyl, Dept. of Obstetrics, Johns Hopkins Hospital, Baltimore, Maryland
F. Carone, Dept. of Pathology, Northwestern Medical School, Chicago, Illinois
~~P. Y-K Wong, Dept. of Medicine, Albany Medical College, Albany, New York~~
S. Hosea, NIAID

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, Maryland

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	0.8	2.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

1. Human urokininogen has been characterized and appears to be a mixture of intact low molecular weight kininogen and breakdown fragments of both high and low molecular weight kininogens.
2. Kininogen has been localized in the distal nephron of the human kidney by immunofluorescence.
3. Kininogen appears to be present in whole human saliva.
4. Plasma high molecular weight kininogen appears to be decreased in certain patients with hereditary angioedema and carcinoid syndrome.
5. Both high molecular weight kininogen and prekallikrein appear to decrease in the plasma of pregnant women immediately after parturition.
6. In both human urine and human parotid saliva, glandular kallikrein increases in normal volunteers on low salt diet or after administration of Fludrocortisone.

PHS-6040
(Rev. 10-76)

Objectives: To improve procedures for the assay of components of the kallikrein-kinin-kininogen system and to use these procedures to establish the role of the system in health and disease.

Methods: Bioassay, radioimmunoassay, radiochemical assay, chromagenic substrate assay, chromatographic and electrophoretic techniques.

Major Findings:

Urokininogen: Two kininogen radioimmunoassays (RIAs), one based on an antibody to the unique light chain of HMW kininogen (Z01 HL 01022-02 CH) and one based on an antibody which recognizes the heavy chains of all human kininogens, have been used in conjunction with a kinin RIA to characterize urokininogen. When 24h urines were examined, the daily excretion of heavy chain antigen was about 1,600 μg while about 300 μg of light chain were excreted in 24h. The daily excretion of free kinin was about 8 μg and treatment with trypsin or kallikrein increased the level to about 10 μg. No sex difference was seen for any antigen. Urokininogen was purified by sequential batch immunoaffinity adsorption with immobilized antibody to heavy chain and with immobilized antibody to light chain. The two concentrated eluaks obtained most of the kininogen antigen from urine and significant releasable kinin was also recovered. Gel filtration of the concentrates showed that all releasable kinin was associated with kininogen antigen. When molecular weight studies were performed on the concentrates using SDS-PAGE a wide range of molecular weights were observed. All visible bands were associated with antigenic activity. The concentrate produced by adsorption with antibody to heavy chain (heavy chain concentrate) had major stained and antigenic bands corresponding to intact LMW kininogen and/or its heavy chain while the concentrate produced by adsorption with antibody to light chain (light chain concentrate) appeared to represent predominantly degradation fragments of heavy and light chains. Alkaline-PAGE of the heavy chain concentrate showed a band pattern which was strikingly similar to that of LMW kininogen while the light chain concentrate gave a pattern which, once again, was suggestive of a range of breakdown fragments. Immunofluorescence studies were performed and, using antibody to heavy chain, strong fluorescence was observed in the distal nephron. No fluorescence was observed with antibody to light chain. It is concluded that the kidney is a source of LMW kininogen which may contribute to the levels of urokininogen. The light chain antigen probably represents filtered breakdown products of plasma HMW kininogen and as such the light chain RIA could be of use as a measure of plasma HMW kininogen turnover. Preliminary measurements indicate that kininogen antigen may be present in whole human saliva, although no signigicant antigen was detected in parotid saliva.

Plasma Kininogens: HMW kininogen has been measured in plasmas of patients with hereditary angioedema (HAE), carcinoid syndrome and in pregnant women at childbirth. In both the carcinoid syndrome and HAE studies there were several patients who showed consistently low levels of HMW kininogen. For HAE patients both RIA and bioassay measurements were made and good agreement was seen between the two methods. Preliminary results in the pregnancy study indicate that there may be a decrease in HMW kininogen following parturition.

Plasma Prekallikrein: Depletion of prekallikrein has also been measured in the HAE and pregnancy studies using a chromogenic substrate assay. Preliminary results in the pregnancy study again indicate that levels drop following parturition. Fluctuations in prekallikrein are also seen in some HAE patients but as yet insufficient data exists to correlate these fluctuations with attacks.

Glandular Kallikrein: Studies of urinary kallikrein excretion on variable salt diets were continued and extended. Esterase assays showed that both free and prokallikrein were significantly increased by low salt diet or by administration of Fludrocortisone. Kallikrein antigen as measured by RIA was also increased. The RIA has also been used to study the effects of salt diet on kallikrein excretion by the parotid gland. Once again excretion was increased on low salt diet. Measurements of glandular kallikrein in the plasma of patients with carcinoid syndrome have been performed but results are difficult to interpret due to the non-parallel cross-reaction of plasma in the assay.

Proposed Course: Clinical studies of HAE and pregnancy will be continued. In addition urinary light chain antigen will be measured in patients where plasma HMW kininogen is rapidly turning over to confirm that the urinary antigen is really a metabolite. Human and monkey kidney tissue will be examined for kininogen using an RIA for human serum albumin to account for blood contamination. Studies on kininogen in saliva will also be continued.

Publications:

1. Proud, D., Pierce, J.V., and Pisano, J.J.: Radioimmunoassay of Human High Molecular Weight Kininogen in Normal and Deficient Plasmas. J. Lab. Clin. Med. 95: 563-574, 1980.

2. Corthorn, J., Imanari, T., Yoshida, H., Kaizu, T., Pierce, J.V. and Pisano, J.J.: Isolation of Prokallikrein from Human Urine. Adv. Exp. Med. Biol. Vol. 120B: 575-579, 1979.

3. Vinci, J.M., Zusman, R.M., Izzo, J.L., Jr., Bowden, R.E., Horwitz, D., Pisano, J.J. and Keiser, H.R.: Relationships of Human Urinary and Plasma Kinins to Sodium-Retaining Steroids and Plasma Renin Activity. Adv. Exp. Med. Biol. Vol. 120A: 503-513, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01018-23 CH

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemistry of the Kallikrein-Kininogen-Kinin System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J.V. Pierce, Ph.D.	Research Chemist	CH NHLBI
OTHER:	J.J. Pisano, Ph.D.	Head, Sect. on Physiol. Chem.	CH NHLBI
	Y. Hojima, Ph.D.	Visiting Associate	CH NHLBI
	M. Bribitzer	Chemist	CH NHLBI

COOPERATING UNITS (if any)

J. Finlayson, D. Aronson, and B. Alving, Bureau of Biologics FDA, Bethesda, MD; E. Shaw and C. Kettner, Biol. Dept., Brookhaven National Lab., Upton, NY

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

INSTITUTE AND LOCATION
NHLBI, NHI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Two highly specific polypeptide inhibitors of human Hageman factor (Factor XII) fragment(s) (HF_f) were purified from corn seeds (M_r 12 000; pI 5.1, 6.3, and 7.7) and from pumpkin seeds (M_r 4 000; pI 8.3). They form 1:1 molar complexes with HF_f and bovine trypsin, are "arginine" inhibitors, markedly prolong the activated partial thromboplastin time of human plasma, and do not inhibit human plasma or urinary kallikreins, plasmin, α-thrombin, hog pancreatic kallikrein, bovine Factor Xa, or α-chymotrypsin.

Human plasma prokallikrein activated briefly by catalytic amounts of HF_f gave a heavy chain of M_r 53 000 and two light chains of M_r 40 000 and 37 000, corresponding to the 88 000- and 85 000-dalton proenzyme forms. Further incubation after inhibiting HF_f by excess corn inhibitor converted the M_r 53 000 chain into 33 000- and 20 000-dalton fragments.

Objectives: Purification of glandular kallikreins and prokallikreins and of components of the plasma kinin, clotting, and fibrinolytic systems for purposes of characterization and production of specific antiserums. Preparation of purified specific antibodies and isolation from plant and animal sources of specific inhibitors for human plasma proteases (kallikrein, Hageman factor, plasmin, thrombin, Factor XI, etc.) for biochemical, clinical and other studies. Preparation of affinity adsorbents from purified antibodies, antigens, enzymes, and inhibitors for purification and other purposes, such as devising specific biochemical and radioimmunochemical assays. Application of these purified materials, affinity adsorbents, and assay methods to studies of normal and pathological states in man and other primates.

Major Findings:

1. Human Plasma Kallikrein and Prokallikrein. Using discontinuous SDS-PAGE with reductant, we have found Mr values of 88 000 and 85 000 for the two molecular weight forms of plasma prokallikrein (Previously we had found M_rs of 84 000 and 81 000 using SDS-PAGE without reductant: see Z01 HL 01018-22 CH). The ratio of these forms in the four isoelectric forms, pI 8.5, 8.8, 9.1, and 9.3, has been estimated by scanning stained SDS-PAGE disc gels to be 95:5, 84:16, 67:33, and 51:49, respectively. Activation of the four pI forms of prokallikrein by catalytic amounts of HF_f for several minutes at ~25° gave protein bands with M_rs of 53 000, 40 000, and 37 000. The ratio of the 40 000 to 37 000 bands correlates well with the 88 000:85 000 ratios given above for the four pI forms of prokallikrein. Further incubation of prokallikrein and HF_f in the presence of excess CHFI (to block further action of HF_f) converts the 53 000-dalton chain into two chains of 33 000 and 20 000, suggesting that limited autolysis has occurred. Mandle and Kaplan (J. Biol. Chem, 252, 6097-6104, 1977) have reported that the active center Ser residue is located in the light chains.

2. Hageman Factor Fragment(s) (HF_f). Besides the highly specific pumpkin and corn inhibitors of HF_f described below, we have tested the reactivity, k_{app}/I (which approximates k_2/K_i when $K_i >> I$), of 10 peptides of Arg and Lys chloromethyl ketones synthesized by Drs. C. Kettner and E. Shaw. While Dns-Ala-Phe-Arg-CH_2Cl was 144 times as effective in inhibiting plasma kallikrein as HF_f ($k_{app}/I = 4.1$ and 0.17 $mM^{-1} min^{-1}$ at 0.05 and 1.0 μM concentrations of chloromethyl ketone, respectively), no chloromethyl ketone has yet been found giving the converse situation.

3. Plant Inhibitors of HF_f, Plasma Kallikrein, Human Urinary Kallikrein, and Trypsin. (a) Pumpkin HF_f Inhibitor (PHFI). The pumpkin seed inhibitor of HF_f mentioned in Z01 HL 01018-22 CH has been purified 1,800-fold in 15% yield by acetone fractionation, DEAE-cellulose and CM-Sephadex A-25 chromatography, and Sephadex G-50 gel filtration. A pI of 8.3 was found by sucrose density gradient isoelectric focusing. The M_r values estimated by Sephadex G-25 gel filtration, SDS-PAGE, and amino acid anlysis are 4 000, 4 200, and 3 320, respectively. Amino acid analysis of PHFI (assuming 2 mol Val/mol PHFI) gave $Asp_2Ser_1Glu_2Pro_1Gly_2Ala_1Cys_3Val_2Met_1Ile_1Leu_3Tyr_1Lys_3His_1Arg_2$ and

no Thr, Phe, or Trp residues (<0.1 mol/mol of inhibitor). Assuming an M_r of 4 000, the K_i values of PHFI with trypsin and HF_f are 1.1×10^{-8} M and 3.3×10^{-8} M, respectively. Acid hydrolysis of dansylated PHFI gave only ε-Dns-Lys, indicating that the α-amino group of the NH_2-terminal amino acid residue is blocked. The COOH-terminal residue was identified as Arg after acid hydrolysis of ^3H_2O-treated PHFI. No carbohydrate was found by the phenol-H_2SO_4 method. Except for M_r and pI, PHFI and CHFI are remarkably similar (see Z01 HL 01018-21 LC): PHFI does not inhibit human plasma or urinary kallikreins, plasmin, α-thrombin, hog pancreatic kallikrein, bovine Factor Xa, or α-chymotrypsin. The inhibitory activity for trypsin of both CHFI and PHFI was destroyed by treatment with 1,2-cyclohexanedione but not with trinitrobenzene-sulfonic acid, indicating that both polypeptides are "arginine" inhibitors. Also, affinity chromatography of both inhibitors on trypsin-agarose columns destroyed ∿ 60% of their activity toward HF_f but had no effect on their activity toward trypsin. On the other hand, no loss of inhibitory activity for HF_f was found after affinity chromatography of PHFI and CHFI on anhydrotrypsin-agarose columns. Finally, PHFI significantly prolongs the activated partial thromboplastin time of normal human plasma.
(b) Lily Bulb Inhibitor of Human Urinary Kallikrein (HUK). The potent trypsin and HUK inhibitor in lily bulb extracts is being purified for use in affinity chromatographic purification of HUK. Affinity chromatography of the lily inhibitor on trypsin-agarose columns showed a high loss of inhibitory activity for HUK but not for trypsin, whereas the use of anhydrotrypsin-agarose gave a good recovery of both activities.

4. Human Plasma Kininogen. We showed previously (Z01 HL 01018-19 LC and Z01 HL 01016-09 CH) that pepsin is a potent kinin-forming enzyme. However, it now appears that pepsin acts only on the type I kininogen sometimes produced during isolation (in this kininogen, the Arg-Ser bond at the COOH-terminus of the bradykinin sequence has been cleaved). No kinin was released when pepsin was incubated with fresh or outdated human plasma at pH 2, although the expected amount of Met-Lys-bradykinin was released by pepsin when purified kininogen containing pepsin substrate was added to these plasmas. Only small amounts of kinin were formed by peptic digestion of plasma stored at room temperature for several seeks. Pre-incubation of highly purified Prep B kininogens at pH 6-8 and 25-37° often gave much larger amounts of pepsin substrate.

Sucrose density isoelectric focusing of Prep B kininogens at 4° initially gave at least five sharp bands of precipitate in the pH 5.0 region. Unfortunately, by the time fractions were taken, the precipitates had flocculated and obliterated the separation of bands (and also tended to stick to the walls of the electrofocusing apparatus when the column was emptied), so that the initial separation was largely destroyed. In a second experiment, the section of the gradient containing the precipitated bands was removed from the column soon after they had appeared. Treatment of this fraction with more ampholyte, as well as with Triton X-100, dissolved most of the precipitate. However, when the supernate of the centrifuged fraction was returned to the apparatus and electrofocusing was resumed, the bands of precipitate reformed. Electrofocusing of Prep B kininogens in polyacrylamide gels under various conditions indicated that kininogens at their pIs might be more soluble in glycerol than in sucrose. The nonionic detergent BRIJ-58, used by others to avoid isoelectric precipitation, in the present case enhanced rather than prevented precipitation.

Proposed Course of Project: Human Plasma Kallikrein and Prokallikrein. We plan to determine the nature of the difference between the 85 000- and 88 000-dalton forms of prokallikrein and to test whether the former is derived from the latter by limited proteolysis. To facilitate such studies, we hope to separate these two molecular weight forms.

Hageman Factor. Further kinetic studies of the inhibition of HF_f, as well as plasma kallikrein, by peptide chloromethyl ketones and by other synthetic active site inhibitors will be done.

Plant Inhibitors. Work is in progress to recover pure native inhibitors from pumpkin and corn seeds and from lily bulbs by affinity chromatography on anhydrotrypsin-agarose columns.

Kininogen. Further attempts to resolve the isoelectric forms of kininogen in plasma and in purified fractions will be made for purposes of comparing these forms with those previously obtained by DEAE-cellulose chromatography and determining if they are present in fresh plasma treated in various ways designed to enhance or to prevent limited proteolysis.

Publications:

Proud, D., Pierce, J.V., and Pisano, J.J.: Radioimmunoassay of human high molecular weight kininogen in normal and deficient plasmas. J. Lab. Clin. Med. 95: 563-574, 1980.

Ørstavik, T.B., Brandtzaeg, P., Nustad, K., and Pierce, J.V.: Immunohistochemical localization of kallikrein in human pancreas and salivary glands. J. Histochem. Cytochem. 28: 557-562, 1980.

Kettner, C., Mirabelli, C., Pierce, J.V., and Shaw, E.: Active site mapping of human and rat urinary kallikreins by peptidyl chloromethyl ketones. Arch. Biochem. Biophys. 202, 1980 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01024-1 CH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Origin and Significance of Immunoreactive Glandular Kallikrein in Rat Plasma

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John J. Pisano	Head, Sec. on Physiological Chemistry	CH NHLBI
OTHER:	David Proud	Visiting Fellow	CH NHLBI
	Jack V. Pierce	Research Chemist	CH NHLBI
	Marian E. Warner	Biologist	HE NHLBI
	Harry Keiser	Deputy Chief, Hypertension-Endocrine Branch	HE NHLBI

COOPERATING UNITS (if any)

Hypertension-Endocrine Branch
W.J. Lawton, V.A. Hospital, Iowa City, Iowa
F. Carone and S. Nakamura, Northwestern University School of Medicine, Chicago, IL

LAB/BRANCH
Laboratory of Chemistry

SECTION
Section on Physiological Chemistry

INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Immunoreactive glandular kallikrein has been detected in rat plasma using a radioimmunoassay (RIA) for rat urinary kallikrein (RUK). Gel filtration of rat plasma revealed three peaks of antigen. Only the third peak (Peak 3) showed immunological identity to RUK. Bilateral nephrectomy significantly ($p<.001$) increased the antigenic contents of whole plasma and of Peak 3. No significant difference in either whole plasma antigen or gel filtration profile was seen when pancreatectomized rats were compared to sham-operated controls, but both whole plasma antigen and the antigenic content of Peak 3 were significantly reduced ($p<.001$) after bilateral submandibular/sublingual gland excision. Peak 3 was indistinguishable from purified rat glandular kallikreins on gel filtration. Purified Peak 3, however, had no activity against kininogen or the chromogenic substrate S-2266, and showed a consistently smaller molecular weight (29,500) on SDS-polyacrylamide gel electrophoresis than that of RUK (32,000). Hence, glandular kallikrein circulates in rat plasma in an inactive form. The submandibular and sublingual glands appear to be a major source of the plasma antigen and the kidney plays an important role in the clearance and/or metabolism of glandular kallikrein from plasma.

Objectives: To continue studies to characterize glandular kallikrein in rat plasma and to determine its origin(s).

Methods: Radioimmunoassay for rat glandular kallikrein (Project No. Z01 HL 01927-01 HE). Surgical removal of organ beds. Gel filtration and ion-exchange chromatography. Assays for functional kallikrein using a chromogenic substrate or bioassay of kinin released from kininogen.

Major Findings: Immunoreactive glandular kallikrein has been detected in rat plasma. Although rat plasma did not cross-react in a parallel manner in the RIA, a highly significant ($p<.001$) 4-fold increase in antigen was seen after bilateral nephrectomy. To further investigate this finding plasmas were gel filtered and three peaks of antigen were detected in both sham-operated and nephrectomized rats. The first two peaks appear to represent inhibitor complexes and the antigenic contents of these peaks were similar in both nephrectomized and sham-operated animals. In contrast, the third peak showed immunological identity to rat urinary kallikrein (RUK) and the antigenic content of this peak was increased about 6-fold in nephrectomized rats compared to sham-operated controls, hence supporting the data obtained with whole plasmas.

Further evidence that the kidney is not a major source of glandular kallikrein in rat plasma comes from measurements made on rat renal lymph and on plasmas obtained simultaneously from a renal artery and a renal vein. Although these results cannot be regarded as definitive, due to the non-parallel cross-reaction of plasma, no arterio-venous difference could be detected across the kidney and renal lymph contained levels of antigen which were not significantly greater than those detected in plasma.

In pancreatectomized rats the antigenic content of whole plasma and the profile obtained after gel filtration did not differ from those of sham-operated controls. Bilateral submandibular/sublingual gland excision, however, caused significant reduction in whole plasma antigen levels and the antigenic content of Peak 3 when compared to controls. Bilateral submandibular/sublingual gland excision was performed in conjunction with bilateral nephrectomy. The increases in whole plasma antigen and in Peak 3 which were produced by nephrectomy alone were not seen after the combined surgery.

Peak 3 was indistinguishable from purified RUK and rat submandibular kallikrein on gel filtration and behaved like a typical glandular kallikrein on ion-exchange chromatography. Purified Peak 3, however, had no activity against kininogen or the chromogenic substrate S-2266, and showed a consistently smaller molecular weight (29 500) on SDS-polyacrylamide gel electrophoresis than that of RUK (32 000). Peak 3 could not be activated by trypsin.

Hence Peak 3 appears to represent glandular kallikrein which as been inactivated in such a way as to leave the molecule almost intact and immunologically unchanged. The submandibular and sublingual glands appear to be the major source of this antigen. It is not known if the antigen enters the venous effluents of the glands and is inactivated after entering the circulation or if the enzyme is swallowed in saliva, inactivated in the stomach and then absorbed across the intestine. Ultimately, the antigen is cleared by the kidney where it may contribute to urinary antigen levels but not to levels measured by esterase or biological activity.

Proposed Course: No further studies are planned.

Annual Report of the
Clinical Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for the major red cell disorders. Red cell diseases which produce significant morbidity and mortality include thalassemia, severe hemoglobinopathies of which sickle cell anemia is the most common, and the various syndromes of bone marrow failure. In our laboratory investigations, we attempt to focus the most advanced techniques and conceptual knowledge on several problems. These include analysis of the differentiation and maturation of erythroid stem cells, elucidation of globin gene structure and expression, and investigation of the phenomenon of hemoglobin switching whereby one hemoglobin is replaced by another during normal development. Our clinical projects are mainly directed toward devising or applying new therapeutic strategies. Many patients with chronic anemia require regular blood transfusions and often develop pathological iron overload. Efforts are directed toward defining the extent of organ dysfunction due to iron and to accessing the potential benefit of iron chelation in these patients. A randomized trial comparing various methods of immunosuppressive therapy is being conducted in patients who have bone marrow failure which may be immunologically mediated.

Much of the work in the Clinical Hematology Branch relates to the general problem of hemoglobin switching. In man and other species, embryonic hemoglobin-produced early in gestation - is replaced by fetal hemoglobin which is the predominant oxygen carrying pigment in circulating red cells during the latter two thirds of gestation. Around the time of birth, this fetal hemoglobin is replaced by adult hemoglobin. The hemoglobin molecule is a tetramer composed of four polypeptides. In human adult hemoglobin (Hb A) two are α and two are β globin while in fetal hemoglobin (Hb F), two α globin chains combine with two γ globin chains. The human β and γ globins are encoded by closely linked genes on chromosome 11, whereas the α globin genes lie on chromosome 16. Thus the switch from Hb F to Hb A reflects selective expression of the closely linked and structurally analogous γ and β globin genes. Our efforts have focused on elucidation of the manner in which this development switch in hemoglobin synthesis is regulated with the goal of attempting to devise some means to increase fetal hemoglobin synthesis in patients with severe disorders of hemoglobin structure or production.

The recombinant DNA and molecular cloning techniques provide powerful tools to investigate gene structure and organization. Studies by other workers have shown that the β-like globin gene cluster includes two γ genes, a δ gene, and a β gene arranged in that order on the chromosome and separated by 3.5, 13.5, and 4.5 kilobases (kb) of DNA, respectively. Our studies of the genomic region which includes this gene cluster have led to the isolation of a number of recombinant bacteriophage containing β-like genes. (Individual project report: Characterization of the DNA sequences surrounding the human β-like globin genes). We have found that the ε gene which encodes for an embry-

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onic β like globin is 14 kb from one of the two γ globin genes. The γ and β globin genes, although they presumably arose by a gene duplication event, are now embedded in DNA regions which bear little homology to one another. A 6.4 kb segment of DNA downstream from the β globin gene was found to be one member of a family of moderately repetitive DNA sequences which constitutes 0.5 - 1.0% of the entire human genome. These examples illustrate the manner in which the molecular cloning techniques have facilitated definition of the anatomy of the globin gene region. Our goals now include the definition of the function of the repetitive DNA sequences. Also we hope to obtain a transcriptional map of the entire region thereby learning whether other expressed structural genes are included.

The thalassemias are congenital anemias characterized by deficient synthesis of one of the globin components of the hemoglobin molecule. In β thalassemia, a small amount of β globin may be produced or alternatively - β globin synthesis may be completely absent. Thus these disorders represent examples of regulatory mutations. Our analysis is focused on identifying the molecular lesions in patients with these disorders with the anticipation that insights into normal gene regulation may be achieved (Individual project report: "Molecular defect in β thalassemia"). Knowledge of normal gene structure has facilitated our study of β thalassemic mutations. All normal globin genes include two introns or intervening sequences which divide the coding parts of the gene into three segments. In the human β gene, the smaller intron is approximately 100 nucleotides in length while the larger is 850 nucleotides. We have identified an 1800 nucleotide globin mRNA precursor in human bone marrow cells which represents an RNA transcript containing sequences copied from the coding blocks and the two introns. This precursor is analogous to those previously identified for mouse and rabbit β globin mRNA. Processing of the precursor to mature mRNA involves excision of the intervening sequence RNA and precisely correct ligation of the mRNA coding sequences to give a functional mRNA molecule. The precursor RNA is thought to include signals in the form of specific sequences which allows this precise splicing to occur. Support for the hypothesis that the β thalassemias may represent mutations which affect processing of the β globin mRNA precursor includes our finding that the concentration of precursor molecules is two to three fold higher in bone marrow cells of four patients with β thalassemia than in controls with an equal degree of erythroid hyperplasia. Furthermore, β globin mRNA synthesis was found to be normal relative to α in cells from two thalassemic patients but there was a delay in the transport of newly synthesized β globin mRNA sequences from nucleus to cytoplasm. These two patients bone marrow cells also contained abnormal precursor molecules not found in the controls. Thus these data establish that defects in processing of the β globin mRNA precursor may cause β thalassemia. Further study of these mutations should help identify RNA sequences necessary for normal processing, lead to the elucidation of the normal pathway for removal of intervening sequence RNA and facilitate identification of obligate processing intermediates.

More directly relevant to the problem of hemoglobin switching have been our measurements of the concentration of γ and β globin mRNA precursors in fetal and adult red cells (Individual project report: "Molecular Defect in β Thalassemia"). The large intron of each of the genes is contained in a Bam

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HI: Eco RI fragment which includes very little globin coding sequence. Thus these fragments, when made radioactive, can be used as probes in solution hybridization reactions with total cellular RNA to measure the concentration of precursor mRNA specifically. We have found that the concentrations of mature γ and β mRNA in cells from the fetal and adult developmental periods are roughly proportional to the concentration of their precursors. These results suggest that modulation of the level of expression of these genes occurs at the level of transcription. Two β-like genes are expressed in adult human bone marrow cells, those for δ and β globin; these two globins are produced in a ratio of 1:40 respectively. We found that the δ globin mRNA precursor is present in bone marrow cells at only one tenth the concentration of the β globin mRNA precursor suggesting that the widely differing amounts of these two globins in adult red cells is based on different rates of transcription of the two genes.

An experimental animal which is highly suitable for investigation of hemoglobin switching is the sheep for this species exhibits a fetal to adult hemoglobin switch that is quite analogous to that which occurs in man. Furthermore, certain sheep have an additional β globin gene which is specifically activated during anemia, apparently by direct action of the hormone-erythropoietin-on erythroid stem cells. Thus the switch to Hb C production is a readily manipulable phenomenon which can be approached experimentally. Our work using the sheep model is organized among two lines: 1) molecular analysis of globin gene structure and expression in developing erythroblasts and 2) cellular analysis of the commitment to express particular globin genes which occurs in early erythroid stem cells.

The current goal of our molecular analysis of hemoglobin production in sheep is the characterization of the various globin genes (Individual project report: "Regulation of the sheep globin genes"). Our desire is to deduce the general map of the β-like globin gene cluster, to define the distances between the genes, and to define their general structure. In the past year we have performed a detailed comparison of genes encoding for the fetal (γ) and adult (β^A) globin genes. The identity of these genes was established by obtaining the nucleotide sequence for most of their coding blocks. Appropriate transcription and RNA processing signals established these as functional genes for the fetal and adult globins. Nonetheless, the nucleotide sequence upstream from the genes were highly similar in the area which includes the putative promoter for RNA transcription suggesting that selective expression of these genes may not be modulated in this region. Furthermore, the two globin genes are included in 8 kb segments of generally homologous DNA despite their highly selective expression and in sharp contrast to the dishomology found in the flanking regions of the human γ and β globin genes (Individual project report: "Characterization of DNA sequences surrounding the human β-like globin genes"). The sheep globin genes also differ from the globin genes of other species in that they are widely separated on the chromosome, e.g. there is 12-15 kb of DNA on either side of the γ globin gene which does not contain another globin gene. Furthermore, the large introns of the γ and β^A globin genes both contain moderately repetitive DNA sequences. To date we have isolated five additional sheep β-like globin genes in recombinant bacteriophage. These genes either encode for embryonic β-like globins or alternatively they are non-functional or pseudogenes which do not encode for a globin produced at any stage of development. Only in one case was linkage between two separate genes established. Thus although we have already made considerable progress in characterizing the sheep β-like globin gene cluster, much additional work is required before the structural anatomy of this region of the genome is fully defined. Only then will meaningful studies of chromatin structure and the development of a general transcriptional map as outlined in individual project reports "Structure of the Globin Genes in Chromatin" and "Regulation of the Sheep Globin Genes" become possible. Nonetheless the general techniques of molecular cloning and recombinant DNA are sufficiently powerful to obtain the desired information and therefore these studies will be continued.

The second general approach to understanding the regulation of globin genes utilizes culture techniques to examine erythroid stem cells. These cells, present in small numbers, cannot be defined morphologically but can be recognized by the development of progeny colonies in semisolid media. The more primitive of these stem cells (BFU-E) have high proliferative potential and give rise to very large colonies in vitro whereas the more mature stem cells (CFU-E) have lower proliferative potential and give rise to smaller colonies. The erythropoietin induced synthesis of Hb C in sheep has been explored extensively using this methodology (Individual project: "Cellular analysis of hemoglobin switching in sheep"). We have learned that erythroid stem cells are committed early in their development, to give rise to progeny erythroblasts which express the pattern of hemoglobin synthesis determined at the commitment stage. During the past year, analysis of the production of Hb C and Hb F made in single fetal erythroid colonies has established that both types of hemoglobins are synthesized not only in individual bursts but also in the individual sub-colonies of bursts. Thus current evidence suggest that the concentration of erythropoietin to which a stem cell is exposed at a critical phase in its development determines the relative portions of β^A and β^C globin synthesis in its progeny erythroblasts. By obtaining serial bone marrow samples from a young animal following injection of erythropoietin we have found that there is a small change in the proportions of Hb A and Hb C made during the erythroblast maturation phase of red cell production although the primary modulation of this switching phenomenon is clearly at the stem cell stage. The molecular means by which erythropoietin exerts its action is unknown although we have learned that erythropoietin accelerates the rate of cell division and therefore, presumably changes the duration of various parts of the cell cycle during differentiation and maturation of the stem cells into erythroblasts.

Even the earliest stem cells that we are currently able to assay in our in vitro system are already committed with regard to fetal or adult hemoglobin synthesis. Thus erythroid colonies derived from stem cells in fetal tissue make fetal hemoglobin whereas erythroid colonies derived from stem cells in adult tissue make adult hemoglobin. Presumably, the commitment step occurs at some earlier developmental stage than that of the stem cells which are currently forming colonies in our assay system. Thus to study fetal to adult hemoglobin switching at the cellular level in more detail it will be necessary to develop assay systems capable of characterizing even more primitive erythroid stem cells.

The realization that the current assay system for examining erythroid stem cells may not be adequate to add significantly to our knowledge of hemoglobin switching has prompted us to explore other lines of investigation. The general strategy to be utilized is outlined in the individual project report "Use of hybridoma technology in the study of erythroid differentiation". Hybridomas are clones of antibody producing cells derived by fusion of mouse myeloma cells with spleen cells from mice immunized with the antigen of interest. The power of this technology is that the antibodies produced, being the products of progeny cells derived from a single fusion event, have monoclonal specificity. Thus even when a mixture of antigens or a cell population having a diversity of surface determinants are used for immunization, after proper screening of many hybridoma clones, cells producing antibodies with unique specificity can be obtained. For example, although erythropoietin is available only in impure form for use as an immunogen, we are attempting to obtain hybridoma clones producing monospecific antibodies directed toward this hormone. The availability of such an antibody would greatly facilitate the large scale purification of erythropoietin and could also be used in many studies of the mechanism of action and physiology of the hormone. We are also using erythroid cell populations which contain stem cells as immunogens in an effort to obtain antibodies directed toward stem cell surface markers. A number of clones producing antibodies which react with K562 human erythroleukemia cells have been obtained. The sera from the animals which provided the spleen cells used in the cell fusions, does not react with human peripheral blood cells but only reacts with a small fraction of human bone marrow cells making us hopeful that the hybridoma generated antibodies will be relatively uniquely directed toward erythroid stem cell populations. The availability of such antibodies could lead to stem cell purification and further clarification of the role of stem cells at various stages in the developmental pathway in modulating the patterns of hemoglobin switching in man.

Related to these immunological studies of erythroid differentiation are our efforts to define immunologically mediated bone marrow failure syndromes in man to treat these conditions with immunosuppressive therapy. (Individual project report: "Hematopoiesis in Culture"). Sera from patients with pure red cell aplasia contain antibodies which destroy stem cells by a complement mediated mechanism. Such sera might be useful in purifying erythroid stem cells from peripheral blood. Treatment of two patients with pure red cell aplasia with plasmapheresis to remove their serum antibody and with cytoxan and prednisone to suppress the antibody producing cells has resulted in clinical improvement. These patients had not responded to immunosuppresive therapy alone. In light of these initially encouraging results, this clinical protocol will be continued to determine whether this form of therapy will be of long term benefit. Analogous studies in patients with severe aplastic anemia are being conducted although an immunological basis for this disorder remains somewhat conjectural.

The goal of introducing functional genes into the cells of patients with monogenetic mutations and thereby providing genetic therapy for severe homozygous defects seems more realistic based on the recent explosion of knowledge about gene structure and the availability of several strategies to introduce genes into eukaryotic cells. Treatment of homozygous β thalassemia with periodic blood transfusions produces paliation but recent results of our chelation trial (see individual project" "Iron chelation in transfusional hemochroma-

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tosis") suggests that even removal of substantial amounts of iron with subcutaneously administered desferrioxamine may not prevent the development of cardiac complications in such patients. Similarly the currently available strategies for treatment of sickle cell anemia rely only on supportive care and periodic transfusion and thus are not satisfactory. These considerations provided us with a strong impetus to actively pursue the goal of providing gene therapy for these conditions.

Current strategy to introduce genes into cells *in vitro* rely on the use of a selectable marker to insure proliferation of only those cells into which gene transfer has been affected. We are using the gene for the enzyme, dihydrofolate reductase (DHFR). Increased concentrations of this enzyme render cells impervious to otherwise highly toxic concentrations of methotrexate. A hybrid virus has been constructed in which the coding sequences for DHFR replace those for the coat proteins of SV-40. This virus causes an increase in DHFR activity during late lytic infection in permissive monkey kidney cells. Its potential for conferring resistance to methotrexate on non-permissive cells is being explored. Also constructed are recombinants in which the early region promoter of SV40 is used with DHFR coding sequences. Based on current knowledge, these genes have a higher probability of being expressed in cells which are non-permissive for SV-40 infection.

Ultimately the plan is to link a functional DHFR gene with a globin gene and introduce this recombinant into bone marrow cells. Our experiments culminating in the introduction of the human β gene into mouse fibroblasts (Individual project: "Transformation of mammalian cells") has led us to realize that aberrant transcription and a low level of gene expression might be the outcome of introduction of globin genes into bone marrow cells. Remaining to be defined are the necessary DNA sequences which will lead to a high level of expression of the globin gene in the bone marrow cell environment. Therefore, additional recombinants will be constructed which include DNA sequences which may enhance expression of globin genes. These studies are being pursued with the hope that useful genetic therapy will result but with the confidence that meaningful information about the regulation of gene expression will almost certainly be forthcoming.

The several projects within this Branch are related to the biosynthesis of hemoglobin, the regulation of production of specific hemoglobins and the development and differentiation of erythroid stem cells. From this comprehensive approach we hope to provide an experimental basis for optimal therapeutic approaches to various red cell disorders.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-02203-08 CHB

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Defect in Beta Thalassemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Kantor	Visiting Expert	CHB NHLBI
	R. Kaufman	Research Hematologist	CHB NHLBI
OTHER:	P. Turner	Medical Technologist	CHB NHLBI
	M. Pepe	Visiting Fellow	CHB NHLBI
	P. Kretschmer	Visiting Expert	LMH NHLBI
	A. W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)

Laboratory of Molecular Hematology, NHLBI

LAB/BRANCH

Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.75	2.5	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are directed toward determining the molecular lesion affecting β-globin production in patients with homozygous β-thalassemia. Bone marrow cells from four patients with homozygous β^+-thalassemia contained twice as many precursor β globin mRNA molecules than did control cells suggesting that transcription of the β globin gene is normal but that processing of the precursor may be defective. In contrast, the precursor to δ globin mRNA was present in a much lower concentration than β precursor in both control and β-thalassemic cells suggesting that the disparity in the amounts of Hb A and Hb A_2 in human red cells occurs because of different rates of transcription of the δ and β genes. Synthesis of β globin mRNA was found to be normal when β^+-thalassemic bone marrow cells were incubated in vitro with [^{3}H] nucleotides but there was a delay in transport of radioactive globin mRNA sequences from nucleus to cytoplasm again suggesting a processing defect. Finally, RNA from two patients was subjected to electrophoresis in an agarose gel and abnormal precursor molecules were defined by a hybridization reaction. These results suggest that β^+-thalassemia may occur because of mutations which affect RNA processing. Efforts are now directed towards purifying globin genes from patients with processing defects so that the mutations may be defined at the DNA sequence level.

Objectives:

The objective of these studies is to use the naturally occurring model of defective gene expression provided by β-thalassemia to attempt to understand gene regulation. Patients with this disorder make less than adequate quantities of a normal polypeptide and thus may be said to have regulatory mutations. Recognition of certain molecular lesions in this disease may provide clues as to the specific mechanism for regulation of expression of individual genes in human cells. Furthermore, appreciation of the molecular basis of this disorder may suggest an approach to specific therapy.

Methods:

1. Quantitation of globin mRNA precursors: Total cellular RNA is isolated by standard techniques and annealed to a radioactive probe specific for RNA sequences found in the β precursor. This probe is a cloned Bam HI-Eco RI DNA fragment obtained from λHβG1, a recombinant bacteriophage which contains the human δ and β globin genes, and subcloned using the plasmid-pBR322. Formation of radioactive duplex is quantitated by using a single strand specific nuclease or by batch chromatography on hydroxylapatite. Measurement of δ and γ globin mRNA precursor concentrations is by analogous techniques using the cloned large intron fragments from these genes.

2. Analysis of RNA metabolism: Bone marrow cells are fractionated by buoyant density centrifugation to eliminate most of the enucleated red cells. The remaining nucleated cells are incubated in [^{3}H]nucleosides for periods ranging from 20 minutes to 2 hours and then chased in cold uridine for periods up to 20 hours. RNA is isolated by extraction in guanidinium hydrochloride followed by buoyant density centrifugation in cesium chloride. Radioactive globin mRNA sequences are recovered by annealing to cellulose to which has been bound appropriate DNA fragments from recombinant plasmids containing the human α or β genes derived from cDNA. The RNA is eluted and the α or β mRNA sequences are quantitated by determining the fraction which is protected from RNAse digestion by human non-radioactive globin cDNA.

3. Characterization of globin mRNA precursors: Nuclear or total polyadenylated RNA from human bone marrow cells is fractionated by electrophoresis in agarose gels containing methyl mercury. The RNA is transferred to diazotized cellulose paper and annealed to probes specific for either intron or coding sequences.

4. Molecular cloning of human globin genes: High molecular weight human DNA is partially digested with Eco RI. A fraction corresponding to 10-20 kilobases (kb) in length is purified by sucrose gradient centrifugation and ligated to the purified arms of Charon 4A and cloned into E. Coli. Alternatively, high molecular weight human DNA is digested with Hind III, size fractionated by agarose gel electrophoresis to obtain fragments ranging from 7-9 kb in length, ligated to a plasmid vector linearized with Hind III, and cloned into E. Coli. Recombinants containing globin gene sequences are identified by plaque or colony hybridization using a probe specific for human β globin genes.

5. Restriction endonuclease mapping: High molecular weight DNA is prepared from peripheral blood leukocytes by proteinase K digestion, phenol extraction, dialysis, RNAse digestion, phenol extraction and a final dialysis step. This high molecular weight purified DNA is digested with specific restriction endonucleases, fractionated by agarose gel electrophoresis, and transferred to a nitrocellulose filter by "blotting" and the fragments containing globin gene sequences or other specific sequences are identified by annealing the filter to a radioactive probe followed by radioautography.

Major Findings:

1. Globin mRNA precursor concentration: The large introns of the γ, δ, and β globin genes may be readily isolated from recombinants containing these genes as Bam HI:Eco RI fragments. Because the intron sequences are represented in precursor mRNA molecules but not in the mature mRNA, these introns may be used as specific probes to measure precursor mRNA concentrations. Using this approach we have shown that the β globin mRNA precursor is present at a two-fold higher concentration in bone marrow cells of patients with homozygous β^+-thalassemia than in cells from controls. In contrast, the concentration of δ globin mRNA precursor is only 10-15% of that of β globin mRNA precursor suggesting that differing rates of transcription of the δ and β globin genes is responsible for the widely different accumulation of Hb A_2 ($\alpha_2\delta_2$) and Hb A ($\alpha_2\beta_2$) in human red cells. We have also compared the relative concentration of the γ and β globin mRNA precursors in human fetal and adult erythroid cells. The concentrations of mature γ and β mRNA are roughly proportional to the concentration of their precursors suggesting that modulation of the level of expression of these genes occurs at the level of transcription.

2. Analysis of β globin mRNA metabolism: During a two hour pulse, we have found that the relative synthesis of β and α mRNA sequences is comparable in bone marrow cells from patients with homozygous β^+-thalassemia and from controls with erythroid hyperplasia. However, a substantially higher fraction of radioactive β globin mRNA sequences remain in the nucleus in the thalassemic erythroid cells at two hours than in the control cells. Once the β globin mRNA reaches the cytoplasm in the β^+-thalassemia cells, it appears to be stable.

3. Characterization of globin mRNA precursors: Normal human erythroid cells were found to contain 1800 nucleotide precursors for the δ and β globin mRNAs. An additional 1300 nucleotide RNA species was found in the cells of a Greek patient who was homozygous for β^+-thalassemia; part but not all, of the RNA sequences transcribed from the large intron have been removed from this RNA species. In contrast, the cells of a black patient with homozygous β^+-thalassemia were found to contain a 600-700 bp RNA species which includes RNA sequences transcribed from the large intron and also a small amount of β globin coding sequence. We hypothesized that this species represents a relatively stable product of an abnormally processed precursor molecule.

4. Restriction endonuclease mapping: DNA from a patient homozygous for pancellular hereditary persistence of fetal hemoglobin (HPFH) has been studied to determine the extent the deletion involving the δ and β globin genes. The

5' end of this deletion begins at a point 3-4 kb upstream from the δ globin gene as shown by Southern blot analysis using a cloned unique sequence probe for this region of the genome. Determination of the 3' end of the deletion has been delayed because of the moderately repetitive DNA sequences found 3' to the β globin gene (see project "Characterization of DNA sequences surrounding the human β-like globin genes"). Recently, a cloned DNA fragment derived from DNA 15 kb downstream from the β globin gene has been shown to contain sequences which are unique in the human genome. This fragment should be useful as a probe to define the extent of deletion in our pancellular HPFH patient.

5. Molecular cloning of β-thalassemia globin genes: For unknown reasons, we have been unable to obtain an intact β globin gene from our libraries of cloned human DNA fragments constructed using DNA from thalassemic individuals. These libraries, constructed by partial Eco RI digestion of human genomic DNA, have yielded several recombinants containing human globin genes which have been useful in characterization of the human β globin gene region (see project "Characterization of the DNA sequences surrounding the human β-like globin genes"). However, to increase the probability of obtaining an intact β globin gene from thalassemic DNA, we are modifying the technology of cloning so as to use size selected Hind III fragments which must contain the intact β gene. Screening will be done with the β globin gene large intron as a probe since its sequences are totally unique to the β globin gene region.

Significance to Biomedical Research and the Institute Program:

Homozygous β-thalassemia is a disease which causes severe morbidity and mortality to its victims. Thus an understanding of the genetic basis of this disorder may provide a basis for designing therapy which could be of extraordinary benefit to these individuals. Furthermore, this disease is a prototype of a human regulatory genetic mutation and thus may provide insight into the mechanism of gene regulation in human cells.

Proposed Course of the Project:

Our immediate goals include definition of the normal processing intermediates for the human globin mRNA precursors by use of techniques which allow definition of specific splice sites used in the removal of intervening sequence RNA. We hope to study RNA from the cells of a number of patients with homozygous β^+-thalassemia thereby expanding our knowledge of the processing defects occurring in this condition. These analysis may provide insight into the processing pathway and indicate whether certain splicing sites are obligatory for production of normal amounts of mature β globin mRNA. We intend to continue our efforts to obtain pure globin genes affected by β^+-thalassemia mutation by using the molecular cloning recombinant DNA techniques. Sequencing of specific splice sites may allow definition of the exact mutations which result in abnormal processing. Insertion of large introns from the β globin gene into SV40 vectors may facilitate definition of processing intermediates once the cloned thalassemic genes are obtained.

Publications:

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2. Nienhuis, A.W., Benz, E.J., Jr., Propper, R., Corash, L., Henry, W., Borer, J., and Anderson, W.F. Thalassemia Major: Molecular and Clinical Aspects. Ann of Intern. Med. 91:883-897, 1979.

3. Kantor, J.A., Turner, P., and Nienhuis, A.W. Beta-Thalassemia: Mutations which affect processing of the β globin mRNA precursor. Cell. In Press, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 H1 02204-08 CHB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cellular Analysis of Hemoglobin Switching in Sheep

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. Barker	Research Geneticist (Cell Biology)	CHB NHLBI
Other:	J.E. Pierce	Chief, Laboratory of Animal Surgery	NHLBI
	L. Stuart	Chief, Ungulate Section, NIH Animal Center	NHLBI
	D. Buckhold	Veterinary Officer	NHLBI
	J.A. Kantor	Visiting Expert	CHB NHLBI
	A.W. Nienhuis	Chief, Clinical Hematology Branch	CHB NHLBI

COOPERATING UNITS (if any)
Section on Laboratory Medicine and Surgery, NHLBI;
Ungulate Section, NIH Animal Center; Patient Services Department

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.25	0.25

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of these studies is to establish the basic developmental and cellular events operating in regulating the genes for fetal (γ) and adult (β^A and β^C) globin in sheep erythroid cells. Induction of Hb C synthesis may be achieved in vitro or in vivo by exposure of erythroid stem cells to high erythropoietin (epo) concentration. The amount of Hb C synthesis appears to be related to the degree of differentiation of stem cells which give rise to erythroid colonies in vitro; the earliest stem cells give rise to colonies making more Hb C. We have now investigated the possibility of a second level of control by a cellular mechansim; namely, modulation of the amount of Hb C produced during terminal maturation of erythroblasts. A young adult animal was injected with epo and bone marrow samples obtained at intervals thereafter were fractionated into young, intermediate, and mature erythroblasts. There was a small increase in the proportion of Hb C produced during maturation of the bone marrow erythroblasts derived from stem cells committed by exposure to epo. In other experiments, an assay was devised to quantitate hemoglobin synthesis in individual fetal erythroid colonies in vitro. All colonies examined were shown to be making both Hb F and Hb C; a clonal or stem cell selection mechanism for regulation of Hb C was excluded.

Objectives

The primary objective of this project is to determine the normal mechanism of regulation of expression of globin genes during ontogeny in sheep. Specifically, we wish to learn the relative importance of stem cell commitment with respect to production of hemoglobin in progeny erythroblasts versus changes in the proportion of specific hemoglobins synthesized during erythroid maturation in determining the ultimate proportions of various hemoglobins in red cells.

The globin genes of sheep serve as a suitable model for investigating these phenomena since the several individual globin genes are expressed only during specific periods. Thus the γ globin gene (Hb F = $\alpha_2\gamma_2$) is expressed during fetal life while the allelic adult β globin genes (β^A and β^B) are expressed in adult animals making the normal adult hemoglobins, Hb A ($\alpha_2\beta_2^A$) and Hb B ($\alpha_2\beta_2^B$). The β^C globin gene (Hb C = $\alpha_2\beta_2^C$) is expressed transiently in newborn animals and in adults made anemic. Induction of β^C globin synthsis is specifically stimulated by erythropoietin (epo) both in vivo and in vitro. This readily manipulable switch in hemoglobin production, namely the epo induced synthesis of the Hb C, has served as a focus for our studies.

Methods

1. Erythroid cells are collected from fetal liver or bone marrow of young lambs. For specific studies, the young lambs may be injected with epo and serial samples of bone marrow obtained. The cells are fractionated by centrifugation in Ficoll-metrizamide solution. Further fractionation of cells to obtain fractions with varying degrees of maturation is accomplished by unit gravity sedimentation through a 0.5-2% bovine serum albumin gradient. Cells are grown in plasma clot or methylcellulose cultures with varying concentrations of epo. Individual colonies for analysis are plucked from methylcellulose with a finely drawn capillary pipet. Globin synthesis is measured by exposure of colonies to [^{3}H]leucine *in vitro* followed by chromatographic separation of the globins on an ion exchange column.
2. Analysis of the specific mRNAs contained in individual erythroid colonies is by *in-situ* hybridization. Used as probes are restriction endonuclease fragments from recombinant plasmids into which had been inserted synthetic copies of either the β^B, β^C, or γ globin genes. The colonies are fixed, annealed *in-situ* to the appropriate radiolabelled DNA fragment, and the signal is detected by radioautography.

Major Findings

1. Characterization of erythroid stem cells: Cells from livers of 60-70 day fetuses were cultured at a high concentration of epo in plasma clots. The appearance of erythroid colonies was monitored by microscopy of plasma clots fixed and stained on successive days. Two populations of colonies were detected. The first matured after 4-5 days and then disappeared as the second population appeared at 4 days and was completely hemoglobinized by 8 days *in vitro*. Colonies mature at 4-5 days were small and single while the *second set* of colonies consisted of macroscopic clumps of 2-6 subcolonies. The two types of colonies are derived from stem cells referred to as CFU-E and BFU-E, respectively. BFU-E are thought to be earlier stem cells with respect to their level of differentiation and proliferation potential than are CFU-E. The colonies derived from CFU-E produced 20-30% Hb C while colonies derived from BFU-E produced 50-80% Hb C. These results indicate that the potential for Hb C production in erythroblasts is related to the level of differentiation of the stem cells from which these erythroblasts are derived at the time that the inducing stimulus, namely epo, is applied.

2. Ratio of Hb A and Hb C synthesis during erythroid maturation: A lamb was injected with epo and bone marrow samples were obtained at 36, 48, and 60 hours thereafter. These were segregated into early, intermediate and late stage erythroblasts by unit gravity sedimentation. Individual cell fractions were exposed to [^{3}H]leucine for 4 hours. After cell lysis, the globins were analyzed by ion-exchange chromatography. Hb C synthesis was first seen in the early erythroblast population at 36 hours and appeared subsequently in the intermediate and late erythroblasts at 48 and 60 hours. This progression reflects the commitment event occurring in stem cells. Careful analysis suggest that pro-erythroblasts making 20% Hb C at 36 hours become intermediate stage erythroblasts making approximately 35% Hb C at 48 or 60 hours. Thus, there may be modest changes in the proportion of Hb C and Hb A production during the erythroblast maturation.

3. Hemoglobin synthesis in clonal erythroid colonies: Cells from fetal liver were grown in methylcellulose at high concentrations of epo. At 4-6 days in vitro, individual colonies were characterized as to the degree of maturation of their erythroblasts as early (pale white), intermediate (pink), or late (red). Colonies of defined maturational stage were plucked from the medium with a finely drawn capillary pipet. After washing, the colonies were exposed to [^{3}H]leucine for 4-12 hours and the globin synthetic products analyzed by ion-exchange chromatography. The results indicate that the capacity for Hb C synthesis is not restricted to a single population of erythroid stem cells and, therefore, is not clonal, since all colonies studied synthesized both Hb F and Hb C in varying proportions. The individual sub-colonies of large bursts had similar ratios of the two hemoglobins. At 5 days in culture, colonies of all maturational stages appeared to be making similar proportions of Hb C and Hb F. However, more mature colonies at a later time in culture made slightly more Hb C than colonies from an earlier time period.

4. Detection of specific mRNAs in erythroid colonies by in-situ hybridization. Plasma clot cultures containing colonies derived from erythroid stem cells present in fetal liver were fixed and annealed to radioactive DNA fragments containing sequences specific for ^{C} or mRNA. Preliminary experiments indicate that hybridization does occur to colonies in-situ and that it may be specific for a particular type of mRNA. These studies will continue using [^{3}] ^{C} probe and [^{14}C] probe in a combined hybridization with subsequent analysis using 2 Kodak films, one to trap the photons emitted from [^{3}H] and the other superimposed on it to monitor the decay of [^{14}C]. In this manner, we hope to analyze and quantitate the appearance of specific mRNAs during erythroid maturation.

Significance to Biomedical Research and the Program in the Institute

Several human anemias (e.g., -thalassemia and sickle cell anemia) are characterized by normal globin synthesis and abnormal globin synthesis or function. A common feature of these anemias is that synthesis of fetal hemoglobins is normal during fetal life. Human hereditary persistence of fetal hemoglobin is a mild anemia and requires minimal clinical management. A rational approach to treatment of anemia involving defects in globin synthesis is, therefore, the therapeutic re-utilization of the fetal gene in adult life. Attempts to describe the basic cellular and hormonal regulation of erythropoiesis in sheep are done with the goal of realizing this possibility.

Proposed Course of Experiments

Our previous data appear to suggest that the stem cells from either fetuses or adult animals are committed with regard to their pattern of hemoglobin synthesis. The recent data regarding the Hb C switch also support commitment at the erythroid stem cell level as the primary mechanism by which the hemoglobin phenotype of the red cell is determined. Therefore, to look into the mechanisms or regulation of hemoglobin synthesis, it will be necessary to develop culture techniques suitable for propagation of stem cells from early developmental stages. Growth factors derived from mononuclear cells, presumably T-lymphocytes, are necessary in other species for development of early stem cells. However, many other interactions are obviously necessary and the techniques suitable for propagation of early hematopoietic stem cells *in vitro* in quantities adequate for biochemical analysis have not yet been achieved. With the anticipated departure of Dr. Barker, the principle investigator, to the Jackson Laboratory at the end of this fiscal year, a reassessment of our approach to the problem of hemoglobin switching seems mandatory. The most readily achievable goal appears to be that of characterizing the non- globin gene region completely by use of the molecular cloning techniques as outlined in project "Regulation of the sheep globin genes." Identification of specific proteins which interact to modulate globin gene expression in erythroid cells would then ba a reasonable goal and may be the most fruitful way to investigate the hemoglobin switching mechanism.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-02206 07 CHB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of the Sheep Globin Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Kretschmer	Visiting Expert	LMH, NHLBI
OTHER:	H. Coon	Biologist	LMH, NHLBI
	A. Davis	Research Assistant	CHB, NHLBI
	M. Harrison	Electronmicroscopy Technician	CHB, NHLBI
	M. Goldsmith	Staff Fellow	CHB, NHLBI
	A. W. Nienhuis	Branch Chief	CHB, NHLBI

COOPERATING UNITS (if any)
Laboratory of Molecular Hematology, NHLBI
Section on Laboratory Medicine and Surgery, NHLBI: Ungulate Section, NIH Animal Center

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
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SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to define the mechanism of regulation of the individual globin genes during erythroid differentiation. A previously constructed library of sheep genomic DNA fragments, cloned into E. coli using the bacteriophage vector - Charon 4A, was screened with probes specific for the β-like globin genes of sheep. Four overlapping clones, the inserted fragments of which include 28 kb of sheep genomic DNA, were all found to contain a sheep γ globin gene identified by DNA sequencing of the coding blocks. The γ gene was compared to the previously isolated gene for β^A globin. Each gene contained two introns, one of 110 bp and the second of approximately 800 bp. Analysis by electron microscopy of heteroduplexes formed between recombinants containing either the γ or β^A globin gene demonstrated that these genes are included in a 8 kb region of general sequence homology. Both the γ and β^A genes were found to include moderately repetitive DNA sequences within their large introns. Several recombinants were identified which contain globin genes which on restriction endonuclease or DNA sequence analysis were identified as either encoding for embryonic β-like globins or alternatively, these may represent pseudogenes, the function of which remains undefined.

Objective

The objective of this project is to determine the normal mechanisms regulating the expression of globin genes in erythroid cells. There are several different levels at which regulation might occur, e.g., gene transcription, nuclear RNA processing, globin messenger RNA translation, or alteration of globin mRNA stability. The globin genes of sheep serve as a suitable model for investigating these phenomena since the several individual globin genes are expressed only under specific conditions. Thus, the gamma globin gene (Hb F - $\alpha_2\gamma_2$) is expressed during fetal life, while the allelic adult beta globin genes (β^A and β^B) are expressed in adult animals making the normal adult hemoglobins, Hb A ($\alpha_2\beta_2{}^A$) and Hb B ($\alpha_2\beta_2{}^B$). The β^C globin gene (Hb C) is expressed transiently in newborn animals and in adults made anemic. Induction of β^C globin synthesis is specifically stimulated by erythropoietin both _in vivo_ and _in vitro_.

Techniques are now available which make it possible to completely define gene structure and to determine the homologous regions surrounding the various globin genes. This experimentation involves the use of recombinant DNA technology and characterization of the isolated recombinants by restriction endonuclease mapping, nucleotide sequencing, and heteroduplex mapping by electron microscopy. Our current goal is to define completely the beta globin region in sheep at the DNA sequence level.

Methods

1. Identification of bacteriophage recombinants containing globin genes and or surrounding sequences: A previously constructed library of sheep genomic DNA fragments of 14-20 kb in length, was screened with probes specific for either globin gene sequences of DNA sequences at the extreme end of previously characterized recombinants containing identified globin genes. The probes are purified by gel electrophoresis and rendered radioactive by nick-translation in the presence of [^{32}P]nucleotide triphosphates. Screening is by plaque hybridization. Positive plaques on the primary screen are picked, and replated at a low density in a secondary screen, the goal of which is to obtain the desired recombinant in pure form. Preliminary characterization of the recombinant clones is by restriction endonuclease digestion, agarose gel electrophoresis, and identification of specific sequences by Southern blot analysis with probes specific for globin gene sequences or DNA sequences flanking globin genes in previously characterized recombinants. In this manner, a restriction endonuclease map of the clone can be deduced.

2. DNA sequence analysis: This high resolution but somewhat laborous technique can be applied to specific fragments of interest; most commonly these are restriction endonuclease fragments which contain globin gene sequences. The goal of this type of analysis is to identify the globin gene represented in a particular recombinant. After a restriction endonuclease map of an individual recombinant is obtained, specific DNA fragments are isolated. Individual fragments are labelled on the ends by the polynucleotide kinase (5') or DNA polymerase I (3') reaction. The labelled fragment is cut with another restriction endonuclease and the two [^{32}P] ends are separated by polyacrylamide gel electrophoresis. The DNA nucleotide sequence can then be obtained by partial chemical degradation of the individual end-labelled fragments followed by polyacrylamide gel electrophoresis and radioautography.

3. Analysis of regions surrounding globin gene sequences: To compare the sequence homology surrounding two individual globin genes, DNA derived from recombinants containing these globin genes can be annealed and the resulting heteroduplexes examined by electron microscopy. Homologous regions form duplexes whereas the non-homologous regions remain single stranded; these can be distinguished in the resulting electron micrographs.

4. Determination of repetitive frequency of DNA sequences contained within specific recombinants: Moderately repetitive DNA sequences interspersed among structural gene can be demonstrated by one of two techniques. Total genomic DNA from sheep is rendered radioactive by a nick-translation reaction and annealed to DNA fragments obtained by restriction endonuclease digestion of DNA from individual clones followed by agarose gel electrophoresis. The intensity of hybridization in a Southern blot analysis, to specific fragments is a measure of the repetitive frequency of sequences within that fragment. Alternatively, the clone may be rendered radioactive, digested with specific restriction endonucleases, the fragments resolved by gel electrophoresis, and the gel blotted against total genomic DNA immobilized on a filter. Again the intensity of the radioautographic signal from specific bands is a measure of the repetitive frequency of that DNA sequence.

Major Findings

1. Characterization of the γ globin gene of sheep: Four overlapping recombinants, the entire length of the inserted DNA fragments of which is 28 kb, were found to contain all or a part of a γ globin gene. No other globin gene was found within this 28 kb of DNA, only 1700 bp of which is occupied by the γ globin gene. The DNA sequence obtained demonstrated that this gene encodes for the previously sequenced γ globin of sheep. The γ globin gene was found to contain two introns, one at a position corresponding to amino acid 29-30 of 110 bp in length and the second, of length approximately 800 bp occurred at a position corresponding to amino acids 103-104.

2. Further characterization of the recombinant containing a sheep β^A globin gene: DNA sequence analysis of the gene contained in $\lambda S\beta^A G21$ definite identified it as encoding for β^A globin. Introns analogous to those found in the γ globin gene were also present in this gene.

3. Comparison of the sheep γ and β^A globin genes: DNA sequence analysis of the 200 bp immediately upstream (5') to the γ and β^A globin genes identified a capping box of homology, the Hogness-Goldberg box thought to be important for RNA polymerase binding, and a further box of sequence homology previously identified upstream from β-like globin genes of other species. In this respect the γ and β^A genes appear to have the functional signals necessary for initiation of transcription. Remarkable sequence homology was defined in this flanking region making it probable but not certain that the regulatory signals responsible for the selective expression of these genes are found outside of the immediate 5' flanking region. Electron microscopic analysis of heteroduplexes formed between recombinants containing either the β^A or γ globin gene demonstrated an extensive region of homology encompassing 8 kb of DNA which included the two globin genes. This region of homology extended 4 kb into the 5' flanking region and 1.5 kb into the 3' flanking region. The homology was nearly perfect, although localized regions of non-homology were identified; two in the 5' flanking region and one in the 3' flanking region. These areas of non-homology might conceivably be important in regulation of the selective expression of these genes.

4. Repetitive DNA sequences in and flanking the sheep globin genes: The large introns of the γ and β^A globin genes were found to include DNA sequences which were moderately repetitive in the sheep genome. Nearly 8,000 copies of a sequence in the γ large intron and approximately 300 copies of a sequence found in the β^A large intron are present. Furthermore, several inverted repetitive sequences were defined in the 3' flanking region of the β^A globin gene by electron microscopy.

5. Characterization of recombinants containing unidentified globin genes: Five recombinant bacteriophage have been identified which contain globin genes, the restriction endonuclease map or limited DNA sequence analysis of which demonstrates that these contain globin genes other than those for the γ, β^A, or β^C globins of sheep. These genes may either encode for embryonic β-like globins or alternatively these may be pseudogenes which do not encode for a globin. Such pseudogenes have been identified in the mouse and human globin gene region of other species. The function of such pseudogenes remains undefined.

Proposed Course of Project

Screening of sheep genomic DNA fragment libraries will be continued in order to obtain clones containing the β^C gene and also in an effort to obtain clones which link up previously identified genes thereby allowing construction of a map of the entire globin gene region. The γ and β^A globin genes will be sequenced in their entirety and thus the nature of the moderately repetitive DNA sequences found within the large introns of these genes will be defined. Heteroduplexes analysis will be pursued in an effort to obtain clues regarding non-homologous regions which may have regulatory function. Comparison of recombinant bacteriophage containing the sheep globin genes and those containing the goat globin genes will be done at the level of electron microscopy in a collaborative project with Dr. Jerry Lingrel and colleagues from the University of Cincinnati. By such an analysis, it will be possible to compare the rates of evolution of the individual globin genes, obtain further clues about regions of potential regulatory function, and obtain data useful in achieving the goal of defining a complete map of the genomic region containing the sheep β-like globin genes.

An immediate goal is to identify repetitive and expressed sequences within the globin gene region. Repetitive elements may have potential regulatory significance or alternatively serve as sites; for initiation of DNA replication. Detection of other genes within the globin gene region would be followed by an attempt to identify the gene products and determine whether these genes are coordinately regulated in a way analogous to the globin genes during erythroid differentiation.

The genes and their flanking regions themselves will be utilized to prepare affinity columns suitable for purifying specific nuclear proteins which may serve as transcriptional regulatory factors. An effort will be made to devise a reconstituted system using the cloned globin genes from genomic DNA, histones, and specific nuclear protein molecules to produce a transcriptionally active chromatin complex.

Publications

1. Benz, E.G., Jr., Kretschmer, P.J., Geist, C.E., Kantor, J.A., Turner, P.H., Nienhuis, A.W.: Hemoglobin switching in sheep: Synthesis, cloning, and characterization of DNA sequences coding for the β^B, β^C, and γ globin mRNAs.
2. Kretschmer, P.J., Coon, H., Kaufman, R.E., Chen, M., Geist, C.E., Nienhuis, A.W.: Hemoglobin switching in sheep: Molecular cloning and characterization of the sheep β^A and β-like embryonic globin genes. J. Biol. Chem. 255:3204-3211, 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02208 06 CHB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Iron Chelation in Transfusional Hemosiderosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A.W. Nienhuis	Branch Chief	CHB NHLBI
	Patricia Griffith	Clinical Nurse Specialist	CHB NHLBI
Other:	M. Leon	Clinical Associate	CHB NHLBI
	W. F. Anderson	Laboratory Chief	LMH NHLBI
	M. Gaul	Clinical Nurse Specialist Montreal Children's Hospital Montreal Quebec, Canada	
	H. Strawczynski	Director, Chronic Care Clinic, Montreal Children's Hospital Montreal, Quebec Canada	

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI; Laboratory of Molecular Hematology, NHLBI; Thalassemic Clinic, Montreal Children's Hospital, Montreal Quebec, Canada

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are designed to evaluate the clinical benefits achieved by iron chelation in patients with chronic iron overload. Desferrioxamine is administered by subcutaneous infusion and iron removed is determined by quantitation of urinary iron excretion and careful recording of the total iron administered by transfusion. Those patients who have no evidence of cardiac disease are randomized to receive either ascorbic acid (3 mg/kg) or placebo. Sixty-one patients are now included in our long-term chelation trial and, of these, forty-nine have been randomized to the ascorbic acid trial. Cardiac function is assessed by 24-hour recordings of the cardiac rhythm, echocardiography, and radionuclide cineangiography. Radionuclide cineangiography provides a very sensitive index of cardiac function in that the majority of patients who have received over 100 units of blood failed to increase their ejection fraction during exercise in the normal fashion. Follow-up radionuclide cineangiograms have been obtained in 19 patients restudied at intervals averaging 17 months. Most patients who had received more than 200 units of blood prior to the first study exhibited cardiac deterioration over the interval between the two studies while those who had received fewer than 200 units exhibited stable cardiac function.

Objectives:

The objectives of these studies are to evaluate available iron chelators to maximize their effectiveness, and to test new chelators as they become available. An effort is being made to develop clinical criteria which will be helpful to determine the efficacy of chronic chelation therapy. A randomized trial of supplemental ascorbic acid has been initiated to assess the value and/or toxicity of this agent in promoting mobilization of iron by desferrioxamine in patients with iron overload.

Methods:

Patient populations which participate in these studies include: 1) patients with transfusion dependent congenital or acquired anemia who require regular blood transfusions to sustain life, and 2) patients with idiopathic hemochromatosis at various stages in the process of iron removal by phlebotomy.

Clinical evaluation of organ function include the following:

1) Heart: Estimate of cardiac size by chest x-ray and electrocardiographic analysis is obtained. Echocardiographic studies are obtained to determine anatomical dimensions of the left ventricle and left ventricular function as assessed by resting ejection fraction. In addition, the configuration of the left ventricle and resting ejection fraction is determined by radionuclide cineangiography. Supine exercise is performed to 70% of maximal predicted heart rate and the ejection fraction is again determined by radionuclide cineangiography. By these methods a detailed analysis of cardiac structure and function is achieved.

2) Endocrine evaluation includes specific testing of the pituitary, thyroid, adrenal, pancreatic islets, and gonad function by baseline measurements and various provocative tests.

3) Liver function is determined by standard clinical testing. In addition, liver biopsies are performed to assess histology and to quantitate liver iron concentration.

4) Serial serum ferritin measurements are obtained to assess the utility of this parameter in estimating total body iron stores and also to follow the course of iron removal.

Major Findings:

1. At the present time, 61 patients with homozygous Beta-Thalassemia are participating in our long-term chelation trial. Of these, 49 have been randomized to the ascorbic acid trial. Most of the others have been excluded because of cardiac disease, although two patients refused this phase of the study. In addition, 13 adults are being followed on chelation therapy. These patients have acquired transfusion dependent anemia of various etiologies.

2. Follow-up cardiac evaluations have been obtained in 19 patients included in the initial series evaluated by radionuclide cineangiography. The average interval between studies was 17 months. Eight of 11 patients who had received more than 200 units of blood prior to the first study, exhibited a significant reduction (greater than 5%) in resting and/or exercise ejection fraction on the second study while only 1 of 8 who had received fewer than 200 units of blood prior to the first study exhibited a significant change. An excellent correlation was observed between the change in resting ejection fraction determined by radionuclide cineangiography and that obtained by echocardiography.

Significance to Biomedical Research and to Institute Program:

Hemosiderosis is a major cause of morbidity and mortality in patients requiring prolonged transfusion therapy. The role of iron chelators in improving the clinical course of these patients must be ascertained.

Proposed Course of the Project:

This project will be continued until a suitable iron chelator is found and evaluated or until the need for transfusion therapy in thalassemia and other congenital hemolytic anemias is removed. The majority of our series of thalassemic patients on the chelation trial have had two or more annual evaluations. Within the next several months we anticipate analysing the follow-up studies and comparing the results to baseline data with particular attention to cardiac function. From this analysis we hope to learn whether there is any difference in the clinical course of the patients on ascorbic acid compared to those on placebo and therefore to determine whether this phase of the trial need be continued.

Publications:

1. Nienhuis, A.W., Griffith, P., Henry, W., Borer, J., Leon, M., Strawczynski, H., and Anderson, W.F.: Evaluation of cardiac function in patients with thalassemia major. Annals N.Y. Acad. Sci. 344:384-396, 1980.

2. Nienhuis, A.W., Benz, E.J., Jr., Propper, R., Corash, L., Anderson, W.F., Henry, W., and Borer, J.: Thalassemia major: molecular and clinical aspects. Ann. Int. Med. 91:833-897, 1979.

3. Nienhuis, A.W., and Propper, R.D.: The thalassemias: disorders of hemoglobin synthesis. In Hematology of Infancy and Childhood. (D.G. Nathan and F. Oski, eds) New York, Saunders, In press, 1980.

4. Leon, M., Borer, J., Bacharach, S., Green, M., Benz, E., Jr., Griffith, P., and Nienhuis, A.: Detection of early cardiac dysfunction in patients with severe Beta-Thalassemia and chronic iron overload. New Eng. J. of Med. 301:1143-1148, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-02300-04 CHB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure of the Globin Genes in Chromatin

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard Croissant	Staff Fellow	CHB NHLBI
OTHER:	Merrill Goldsmith	Staff Fellow	CHB NHLBI
	Neal S. Young	Senior Investigator	CHB NHLBI
	Arthur W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)

Molecular Hematology Laboratory

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.75	1.75	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The goal of this work is to determine the structural organization of the individual globin genes in chromatin. Pancreatic DNAse I and spleen DNAse II are used as probes of the structure of the globin genes and flanking DNA sequences in nuclear chromatin. Ultimately we wish to obtain a fraction of chromatin enriched in transcribed sequences. Current work focuses on the mouse erythroleukemia (MEL) cell model. Earlier studies had shown that the globin genes in nuclei from induced and uninduced MEL cells were equally sensitive to fairly extensive digestion with DNAse I. Our current efforts are focused on attempting to define more subtle differences in chromatin structure using limited DNAse I digestion. If these techniques are successful, similar analysis of the individual globin genes in nuclei from sheep and human erythroid cells will be performed to define changes in chromatin structural domains during development as the individual genes are selectively expressed. Fusion of mouse erythroleukemia cells to human fibroblasts results in stable hybrid cells which include chromosome 11. Induction of cells leads to production of mouse and human β mRNA but the human γ gene is not activated. By probing the chromatin structure in the nuclei of such hybrid cells with DNAse I we have shown that the β but not the γ gene is in an active conformation.

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Objectives:

Only a restricted portion of the total DNA sequences in individual cells are expressed. Primary regulation of these sequences is imposed by their individual structure in chromatin. Chromatin is composed of histones arranged in "nu bodies" which take the form of beads strung on the DNA double helix. Non-histone proteins are also found in chromatin and may be concentrated in the transcriptionally active fraction although they are also associated with "nu bodies". The structural arrangement of DNA sequences in chromatin results in only 6-10% of individual genes being available for transcription in specific differentiated cells. The goal of this project is to determine the structural differences of the individual globin genes in chromatin. Furthermore, we hope to learn the structural basis for inclusion of particular genes into the actively transcribed fraction of nuclear chromatin. Identification of specific proteins may lead us to the putative transcriptional regulatory factors which may determine the structure of the gene in chromatin or modulate its transcriptional rate.

Analysis of the residual DNA remaining after nuclei from induced and uninduced MEL cells had been exposed to extensive digestion with DNAse I, by probing with globin cDNA in a solution hybridization reaction, had lead to the conclusion that the DNAse I sensitivity of the coding regions of the α and β globin genes was not affected by induction. There are several logical extensions of these results which we are now pursuing. First, very limited digestion of nuclei followed by restriction endonuclease digestion of purified DNA allows the preservation or disappearance of specific restriction fragments on a Southern blot analysis to be used as an index of DNAse I action. Such studies are likely to be more sensitive than the previous method of analysis in detecting subtle differences in chromatin structure during induced maturation of MEL cells. Second, by use of specific probes for the coding and immediately flanking regions of the globin genes, obtained from cloned genomic fragments, changes in DNAse I sensitivity due to protein binding at possible regulatory sites may be detected. Third, by use of probes obtained from areas of the chromosome varying distances from the genes, the size of the globin chromatin domain may be defined.

Another approach to the study of the conformation of the globin genes in nuclei is to isolate the transcriptionally active chromatin from a cell producing globin and to study its properties. A method for excising active genes involves mild digestion of chromatin with spleen DNAse II which results in differential solubilization of a fraction of chromatin enriched in sequences which are actively being transcribed. This method has been used successfully to obtain a fraction of chromatin from chicken erythrocyte and mouse erythroleukemia cell nuclei which is enriched in globin genes. Our goal is to apply this methodology to sheep and human erythroid cells. We hope to learn whether the β and γ genes are solubilized simultaneously or whether they are selectively released depending on the level of expression of these genes in erythroid cells from different developmental stages. Furthermore, an ultimate goal is to identify specific non-histone proteins associated with the transcriptionally active fraction of chromatin.

Methods

1. Preparation of nuclei: Currently most studies are done with MEL cells. These are grown in tissue culture flasks under standard techniques. For a single experiment in which approximately 2 x 10^8 cells are required, multiple flasks are grown until the cells reach early log phase. The cells in one half of the flasks are induced by addition of Dimethylsulfoxide (Me_2SO) while the remaining cells serve as uninduced controls. Over 90% of the cells are induced to become benzedine positive. Nuclei are prepared by standard techniques except that the buffers contain 2 mM calcium and 5 mM butyric acid. Purified nuclei are resuspended in buffer containing 20% glycerol, electronically counted, and then divided into aliquots of 5 x 10^7 nuclei and frozen in liquid nitrogen. Nuclei have been used up to 4 months after preparation. Similar techniques will be used to prepare nuclei from sheep and human erythroid cells for analogous experiments.

2. Probe preparation: Because the major focus of the recent experimental work has been the analysis of the β^{maj} gene in mouse, details of the preparation of appropriate probes for this region will be described. A 7 kilobase (kb) fragment containing the entire β gene has been subcloned using a bacterial plasmid. The DNA fragment extends approximately 1000 base pairs (bp) on the 5' side of the coding portion of the globin gene. When the plasmid is digested with a combination of Eco RI, Hind III, and Bam HI, two useful fragments are released. The first, a 1000 bp non-coding 5' fragment stops 70 bp before the point at which initiation of RNA transcription is thought to occur. The second fragment is 800 bp and includes two of the three regions of DNA which encode for the final β globin mRNA. These fragments are resolved by agarose gel electrophoresis, recovered from the gel following digestion with sodium iodide by absorption onto a glass fiber filter, and rendered radioactive by a nick-translation reaction.

3. DNAse I digestion of nuclei and analysis of residual DNA: Nuclei exposed to DNAse I at various concentrations contain a graded series of digestions all of which release less than 2% of the total nuclear DNA. DNA remaining following digestion, as well as DNA from control nuclei, is purified by removal of proteins and RNA. The DNA is then digested with various restriction endonucleases. Specifically, a series of experiments have been performed in which triple digestion with Eco RI, Bam HI, and Hind III, is performed. Restricted DNA samples are electrophoretically resolved in 1% agarose gels and then the DNA is transferred to nitrocellulose filters by the standard Southern blotting technique. The filters are incubated in the presence of specific radioactive probes, and after washing, radioautography is performed to localize those specific fragments containing sequences homologous to the individual probes.

4. Recovery of a transcriptionally active chromatin complex: Nuclei are prepared by standard techniques. DNA is prepared and the histones extracted by standard methods. Electrophoresis of these samples allows determination as to whether protein or DNA has been degraded during the preparation of nuclei. For release of transcriptionally active chromatin, limited digestion with DNAse II is performed and the inactive chromatin fractions are selectively precipitated.

Results

1. DNAse I sensitivity of coding and 5' flanking sequences of the β^{Maj} gene in MEL cell nuclei: The sensitivity of a specific DNA region is estimated by measuring the level of DNAse I digestion at which individual restriction endonuclease generated DNA fragments are no longer detected by Southern blotting. This strategy relies on comparing "depletion points." Although DNAse I cuts only one strand of double stranded DNA, as the enzyme concentration is increased, progressively more single-strand nicks are introduced. At some point, nicks are introduced at close enough proximity in the two DNA strands so that at the low salt conditions of isolation and electrophoresis, the double-stranded segment of DNA between the adjacent nicks is no longer stable. Although at low DNAse I concentrations, the partially nicked DNA remains intact and after restriction endonuclease digestion appears as a discrete band after blotting and hybridization, at higher DNAse I concentrations disruption of individual molecules of the fragment ultimately causes the band to disappear. A direct comparison between the coding and 5' flanking fragments may be made since a probe may be used in the hybridization reaction which anneals to both fragments.

To date, two sets of observations have been obtained. First, the 5' flanking and coding fragments depleted at approximately the same DNAse I concentration in digestions of nuclei from induced and uninduced cells. Second, depletion of both fragments occurs at a much lower enzyme concentration in digestions of nuclei from induced cells compared to those from uninduced cells. Thus, while there appears to be no change in the relative sensitivity of the two fragments during induction compared to one another, there is a marked general increase in sensitivity of that portion of the globin gene domain examined by this experiment, upon induction of MEL cells.

2. DNAse II digestions of sheep fetal liver nuclei: Attempts to obtain transcriptionally active chromatin from fetal liver nuclei have not succeeded. Extensive efforts to find conditions whereby the nuclei may be isolated without significant degradation of DNA or histones have, to date, been unsuccessful. Further experiments will be attempted with nuclei from MEL cells.

3. Analysis of hybrid cells: Nuclei from MEL cell-human fibroblast hybrid cells which contain human chromosome 11, where exposed to DNAse I and the residual DNA was recovered. The human β but not the human γ sequences were destroyed indicating that the selective expression of these genes apparently is based on their structure in chromatin.

Significance to Biomedical Research and Program in the Institute

The mechanisms by which eukaryotic cells differentially expressed specific genes have not yet been defined. Any information which clarifies how chromatin structure relates to gene activity is important to elucidate such mechanisms. The long term goal is to provide insights which may lead to a scheme whereby induction of fetal hemoglobin synthesis may be accomplished in diseased individuals with deficient or abnormal hemoglobin synthesis.

Proposed Course of the Project

To date, considerable difficulty has been experienced in reproducing accurately the DNAse I digestions. Furthermore, exact interpretation of the significance of the "depletion point" in terms of relative sensitivity of two DNA regions is difficult. These difficulties have prompted us to consider alternative strategies directed towards defining the transcriptionally active chromatin within the erythroid cell nuclei. A recently described assay whereby quantitation of polymerase molecules on specific DNA fragments may be accomplished will be adapted in our laboratory to the problem of defining the transcriptional status of the individual globin genes and the flanking DNA regions. A more direct approach to the goal of isolating proteins which have regulatory functions within the globin gene region may be achieved by use of cloned DNA fragments isolated as described in project "Regulation of the sheep globin genes."

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-02304-03 CHB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Hematopoiesis in Culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Neal S. Young	Visiting Expert	CHB	NHLBI
Other:	L. Lee	Biologist	CHB	NHLBI
	T. Spiro	Senior Fellow	BB Dept.	NIH
	H. Klein	Assistant Chief	BB Dept.	NIH
	A.W. Nienhuis	Branch Chief	CHB	NHLBI

COOPERATING UNITS (if any)

Blood Bank, CC, NIH

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The ability to grow myeloid and erythroid hematopoietic cells in vitro has provided a convenient method for the assay and enumeration of human and murine stem cells. The dependence of erythropoietic mouse spleen and fetal liver cells on the hormone erythropoietin has served as a basis for several assays of antibodies to erythropoietin produced by hybridoma cells (see project Z01-HL-02309-01). This report describes the usefulness of cell culture techniques in the evaluation of patients with pure red cell aplasia and aplastic anemia who are treated with immunotherapy, including the drugs cyclophosphamide and prednisone combined with plasmapheresis or lymphocyte depletion. In addition, the method serves as a basis for assessment of cell separation techniques in the partial purification of peripheral blood stem cells.

PHS-6040
(Rev. 10-76)

Objectives:

Because there are a number of diseases which result from destruction of cells in the blood by antibodies, such as idiopathic thrombocytopenic purpura and autoimmune hemolytic anemia, a similar immune-mediated destruction of hematopoietic bone marrow elements has seemed likely. The paradigm of this type of disease is pure red cell aplasia, in which there is an absence of erythroid elements in the bone marrow often associated with a gamma globulin which is lytic to erythroid progenitors. Approximately half of the patients with pure red cell aplasia respond to some form of immunotherapy, such as removal of a thymoma or immunosuppressive drugs.

Aplastic anemia is a more common and devastating disorder characterized by the absence of hematopoietic elements in the bone marrow and peripheral blood pancytopenia. Severe aplastic anemia is fatal to 90% of the patients at 1 year. Some clinical and laboratory findings have suggested the possibility that aplastic anemia, like pure red cell aplasia, may be a disorder mediated by cytotoxic antibodies or lymphocytes. For example, some patients with aplastic anemia recover following immunosuppressive therapy or bone marrow transplantation even when there is no donor bone marrow engraftment.

The measurement in the laboratory of stem cells by *in vitro* techniques has obvious usefulness in the analysis of disorders which primarily affect the bone marrow. First, stem cell assays provide a semi-quantitative means of assessing hematopoietic progenitors. For example, in pure red cell aplasia there is decrease in the number of erythroid stem cells but normal quantities of myeloid stem cells; in aplastic anemia, both erythroid and myeloid elements are severely decreased. Second, the measurement of stem cells over time and with treatment may provide clues to the etiology of these disorders. For example, recovery following immunosuppressive therapy would be expected to be accompanied by an increase in the appropriate stem cells; if the number of stem cells remain constant despite improvement in peripheral blood counts, a defect in the differentiation capability of the precursor pool might be inferred. Third, manipulation of cells in culture, such as the addition or removal of cytotoxic cell populations, may provide experimental clues to the etiology of some forms of bone marrow dysfunction. Fourth, stem cell assays are required for preparation of purified bone marrow or peripheral blood fractions of separated precursors. One such means of separation is the use of automated cell separators.

Methods:

Optimal growth of hematopoietic stem cells is highly dependent on the reagents employed, especially fetal calf serum, and on consistency in technique. Even under optimal conditions, there is a wide range of normal values obtained for human progenitor cells obtained from peripheral blood and bone marrow (see Table I).

There are a large number of reports suggesting a laboratory basis for a suspected immunologic cause of aplastic anemia. Unfortunately, almost all

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the in vitro assays suffer from serious methodologic defects and the difficulty of finding adequate controls. We have employed two in vitro assays for presumed immunologic suppression of normal bone marrow growth. In the first, normal serum in various dilutions is incubated for 30 minutes in the presence of complement and normal bone marrow cells. After washing, the normal marrow cells are cultured and the progenitor cells are assayed. In our experience, sera obtained from patients who have been alloimmunized by frequent transfusion to donor erythrocytes are not inhibitory under these conditions. This assay is a crude means of determining serum inhibitory activity. The second method is co-culture of a patient's bone marrow with normal bone marrow cells. When cells from two normal bone marrow aspirations are co-cultured, there is generally a 20-50% increase in the total number of colonies in comparison to either bone marrow alone. The bone marrow obtained from some patients with aplastic anemia appears to suppress normal bone marrow growth and results in a lower number of colonies. Because of the heterogeneity of the cells present in normal bone marrow, it is impossible to ascribe inhibitory activity to a specific cellular subset or to the direct effect of cytotoxic lymphocytes. As with serum inhibition, it is difficult to control for allogeneic effects in co-culture experiments.

Because of the suggestiveness of the clinical studies in aplastic anemia and the difficulty of performing reliable in vitro assays, a clinical trial of immunotherapy in aplastic anemia appeared warranted to test the hypothesis that the disease is immunologic in character. In addition, the response of patients with pure red cell aplasia, a known immunologic disorder, serves as a convenient control for the effectiveness of treatment as well as offering a new mode of therapy for those patients who are refractory to conventional drugs.

Results:

A. Immunotherapy

Two patients with transfusion dependent, refractory pure red cell aplasia of long duration have been treated with intensive plasmapheresis, cyclophosphamide, and prednisone (see Tables I and II). The first patient had been anemic for 15 years and had a hypoplastic bone marrow. Six weeks following the discontinuation of treatment, she had bone marrow cells present on aspiration for the first time in 5 years. Over the course of the next 3 months, there was approximately a 25% reduction in her transfusion requirement. The second patient had been transfusion dependent for 4 years and had demonstrated only imcomplete and transient responses to prednisone and alkylator therapy. Three weeks following discontinuation of treatment, he developed a reticulocytosis, the hematocrit returned to normal, and erythroid progenitors reappeared in his bone marrow. In both patients, serum inhibitors of erythroid, but not myeloid, bone marrow growth were present; titers of these inhibitors fell dramatically with plasmapheresis.

Four patients with severe aplastic anemia as defined by peripheral blood counts have been treated with either lymphocyte depletion or plasmapheresis in combination with cyclophosphamide and prednisone (see Tables I and II). One patient died of an unrelated cause during therapy. Of the remaining three patients, two have shown no response and the third has demonstrated a moderate increase in bone marrow myelopoietic elements without a change in peripheral blood counts. As shown in Tables I and II, evidence of serum inhibitor and co-culture inhibition were present in some of these patients. The failure of patients with aplastic anemia to respond to treatment suggests that the in vitro immunologic findings may be a secondary finding in this disorder.

B. The Collection of Peripheral Blood Hematopoietic Precursor Cells With the IBM 2997 and the Haemonetics Model 30 Cell Separators

Six normal donors have been passed on either the IBM 2997 or Haemonetics Model 30, in order to compare the collection and concentration capacity of these 2 machines for peripheral blood hematopoietic precursor cells. Blood samples pre- and post- run as well as the Haemonetics 30 leukocyte concentrate and aliquots of the IBM 2997 white cell layer obtained at differing rotational speeds have been tested for CFU-C and BFU-E concentrations. To date, the CFU-C concentrations in Haemonetics 30 leukocyte concentrate has been increased 2, 5, and 6 fold, the BFU-E 2, 4, and 9 fold. The CFU-C concentration in the IBM 2997 white cell layer has been increased maximally by 6, 12, and 15 fold; the BFU-E concentration by 4, 10, and 10 fold. The optimal collection speed probably occurs at the higher RPM tested (950-1150 RPM). Our preliminary data suggest that the IBM 2997 will allow superior quantitative collection of peripheral blood precursor cells.

Proposed Course of Project:

Immunotherapy. Initial results have suggested that plasmapheresis and chemical immunotherapy may be an efficient method for inducing remission in patients with refractory pure red cell aplasia. Two patients who have demonstrated refractoriness to Cytoxan therapy are available for immediate entry into the protocol.

In addition to the 4 patients with aplastic anemia who have been treated by the lymphocyte depletion of plasmapheresis, 2 other patients are in therapy and a further 4-6 patients will be treated in a similar fashion. If there is little evidence of clinical benefit of this treatment, despite laboratory evidence of humoral or cytotoxic suppression, serious doubt will be cast on an immunologic mechanism underlying aplastic anemia.

Stem Cell Separation. Further studies are in progress to define more precisely the quantitative collection capacity of the 2 cell separators, the qualitative characteristics of the stem cells isolated, and the donor pool-size of stem cells in normal humans. Final results will be assessed for efficacy of cell separation as a method of collection of stem cells for "bone marrow" transplantation. In addition, peripheral blood stem cells may serve as immunogens for hybridoma antibody production (see Z01 HL 02309-01).

TABLE I INITIAL DATA

	PB		BM			serum	
PRCA	BFU-E	CFU-C	BFU-E	CFU-E	CFU-C	inhibitor	co-culture
WW	11	ND	no aspirate			62% CFU-E 69% BFU-E	1+ (E only)
PC	5	ND	26	192	ND	30% CFU-E	no
AA							
BvN	3	ND	4			53% CFU-E 53% BFU-E	ND
LP	9	ND	7	--	ND	22% CFU-E 5% BFU-E	ND
MR	1	2	6	192	ND		1+
JT	0	1	0	0	0	47% BFU-E 0 CFU-E 19% CFU-C	2+ (E and M)
JL	2	6					
WJ	2	15	2	4	10		

normal range BM CFU-E 413 ± 293
BFU-E 62 ± 47

PB BFU-E 28 ± 9

Normal range for PB + BM CFU-C not established.

TABLE II. RESULTS OF IMMUNOTHERAPY

		Rx	BM BFU-E	PB BFU-E	BM Morphology	Inhibitor	Clinical
Pure Red Cell Aplasia							
1.	WW	P	19/-	9/11	continued aplastic	62% → 2% (CFU-E) 69% → 20%(BFU-E)	25% ↓ transfusion requirement
2.	PC	P	14/26 more clusters per burst	21/5 larger, redder	conversion to nl	30% → 0% (CFU-E)	nl hematocrit, d/c transfusion
Aplastic Anemia							
1.	BvN	P	-/4	2/3 larger, redder	continued aplastic	53% → 0 → 42%	no change
2.	LP	P					ruptured aortic aneurysm, 2nd wk of therapy
3.	MR	LD	52/192	2/2	↑ myeloid cells		no change
4.	JT	P	0/0	0/0	continued aplastic		septic death, 2 weeks $\bar{P}$ rx
5.	JL	LD	r_x in progress				
6.	WD	P					

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02306-02 CHB

PERIOD COVERED
Ocotober 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Transformation of Mammalian Cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M.J. Chen	Senior Staff Fellow	CHB, NHLBI
Other:	A.W. Nienhuis	Branch Chief	CHB, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.75	0.75	

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The transformation of cultured mammalian cells with defined DNA fragments has been explored as a system for studying eukaryotic gene regulation with a special interest in studying the expression of normal human globin genes and to characterize and ultimately correct the molecular defects affecting globin gene expression in patients with homozygous β-thalassemia. The co-transformation of mouse fibroblasts with herpes simplex thymidine kinase gene and a recombinant bacteriophage, λHβG1, containing both the human δ and β globin genes has resulted in mouse cell lines in which the human globin genes are stably integrated without apparent rearrangement. Analysis of DNA from the transformed cells has identified the several Eco RI restriction endonuclease fragments which contain the globin genes. The human globin genes are expressed in the mouse cells for there are 75-100 copies of β globin mRNA per cell as determined by molecular hybridization analysis. Nearly the entire coding sequence of the globin gene is represented in these transcripts, although the RNA molecules may be slightly shorter than authentic β globin mRNA isolated from human reticulocytes. No human β globin was detected by radioimmunoassay of extracts from the transformed mouse cells.

PHS-6040
(Rev. 10-76)

Objectives:

Due e to the development of DNA splitting and gene cloning techniques, certain specific genes other than those of viral orgin can now be obtained in a homogenous state and in large quantity. Consequently, it is possible to study the organization of such genes by restriction endonuclease mapping and to depict the primary sequence by rapid DNA sequencing techniques. Such study constitutes the first step toward the ultimate goal of understanding the fine regulation of gene expression which in turn is essential for understanding differentiation, development, and cancer.

Herpes simplex viral thymidine kinase gene (Herpes TK gene) has been found to transform thymidine kinase negative mouse fibroblast cells in culture into TK positive transformants, selectable in HAT medium. Co-transformation with a second non-selectable gene is also a frequent occurrence if a DNA fragment including that gene is used along with Herpes TK gene in the transformation experiment. Our studies are directed toward exploring the usefulness of the co-transformation system for studying globin gene regulation. Cloned genomic globin genes are introduced into recipient cells and their expression is studied by examining extracts of the cells for RNA transcripts and globin.

Methods:

1. Preparation of transforming DNA: A bacterial plasmid which contains the Herpes TK gene cloned into E. coli using the plasmid vector-pBR322, provided a source of this gene for transformation. λHβG1 DNA containing the human δ and β globin genes was prepared from isolated bacteriophage by standard techniques.

2. DNA transformation: The DNA of the plasmid containing the Herpes TK gene and λHβG1 DNA were co-precipitated with calcium chloride and used to transform TK^- L-cells. Transformants were selected in HAT media.

3. Identification and characterization of the globin genes in transformed cell lines: High molecular weight DNA was prepared from each transformed cell clone using Blin's procedure, cut with suitable restriction endonucleases, electrophoresed in an agarose gel, and the DNA was transferred to nitrocellulose filter papers using Southern blotting techniques. The filter papers were hybridized with probes specific for globin gene sequences.

4. Characterization of globin mRNA transcripts: RNA was prepared from the transformed cell lines by standard techniques. Quantitation of β globin mRNA sequences in total cellular RNA was accomplished with a single stranded totally pure β globin specific DNA probe by solution hybridization. Sizing of the globin gene transcripts was by the Northern blot procedure whereby RNA molecules are resolved in an agarose gel under denaturing conditions. transferred to diazotized cellulose paper and identified by hybridization to the β globin gene specific probe.

Major Findings:

1. Co-transformation with a low ratio (3:1) of λHβG1 DNA and Herpes TK gene plasmid DNA resulted in nearly a 50% efficiency of incorporation of the

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human δ and β globin genes. Thus, 5 out of 11 stably transformed mouse cell lines contained both genes.

2. Southern blot analysis of DNA extracted from several cell lines resulted in the identification of the characteristic Eco RI fragments derived from λHβG1. These results suggest that the human β and δ globin gene region is replicated intact in the co-transformed cells without significant rearrangement.

3. Analysis of total cellular RNA from two lines indicated molecules containing human β globin mRNA sequences were present at less than 100 copies per cell.

4. Nearly complete protection of the human β globin "cDNA" probe indicated that there is fairly uniform transcription of the coding sequences of the human β globin gene.

5. Northern blot analysis of polyA$^+$ RNA from two cell lines suggest that the human globin gene transcripts are approximately 50 nucleotides shorter than authentic human β globin mRNA.

6. No human β globin was detected in transformed mouse cells using a specific radioimmunoassay.

Significance to Biomedical Research and the Institute Program:

These studies are part of a general effort to identify the regions in human DNA that modulate expression of the human β globin genes. A further goal is characterization of the genetic basis of β-thalassemia by studying defective human globin gene expression in cultured cells.

Proposed Course of Project:

Despite the high frequency of co-transformation and, therefore, successful integration of the human globin genes, the low level of their expression and the apparent incorrect initiation of transcription are discouraging with respect to the potential use of this system for analysis of globin gene regulation. Other approaches are being considered whereby it may be possible to integrate defined DNA segments into mammalian cells. An effort must be made to expand the size of the DNA fragment introduced to increase the possibility that all DNA sequences necessary for normal expression will be included. A further goal is the introduction of such DNA fragments into erythroid cells, specifically the mouse erythroleukemia cell line, for in such cells normal regulation of globin gene expression could be more adequately studied than in fibroblasts.

Publications

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02307-01

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Construction of Hybrid Viruses For Use in Gene Transfer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	A. W. Nienhuis	Branch Chief	CHB NHLBI
	V.P. Setlow	Staff Fellow	CHB NHLBI
Other:	P. Turner	Medical Technologist	CHB NHLBI
	L. Killos	Graduate Student	LMH NHLBI
	W. F. Anderson	Laboratory Chief	LMH NHLBI

COOPERATING UNITS (if any)
Laboratory of Molecular Hematology, NHLBI

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These studies are directed toward three goals. The first is to develop a positive selectable marker gene, that for dihydrofolate reductase (DHFR), which may be used to facilitate DNA transfer into mammalian cells by virtue of its ability to confer resistance to methotrexate. The second is to compare the efficiency of hybrid viruses containing the DHFR gene to that of DNA precipitated by calcium phosphate technique in transforming tissue culture cells in vitro or mouse bone marrow cells in vivo, to methotrexate resistance. The third is to use viral transcription and/or replication signals to construct a DNA fragment containing coding portions of a globin gene which results in substantial globin production in bone marrow cells. To date, we have constructed three potentially functional DHFR genes by using the cloned enzyme coding sequences derived from mRNA with SV40 and/or globin gene transcription and RNA splicing signals. One of the constructs has been put into the late region of a hybrid SV40 virus. During the late lytic infection of monkey kidney cells, two RNA species containing the DHFR enzyme coding sequences are produced and the DHFR activity on a functional assay is 6-fold higher than in control cells.

Objectives:

The goal of correcting human genetic defects which result in severe disease by genetic therapy is becoming an increasingly more realistic objective based on substantial knowledge about gene structure and function derived from exploitation of the molecular cloning recombinant DNA technology. Needed are positive selectable markers which can be used to identify and amplify those cells into which gene transfer has been affected. Although, other positive selectable markers are in various stages of characterization, the marker currently available is the gene for dihydrofolate reductase, (DHFR) an enzyme which confers resistance to methotrexate. We hope to adapt this marker to our purpose by using viral DNA fragments to provide necessary replication and transcription signals. Because SV40 virus has been thoroughly characterized and already used as a vector to introduce genes into eukaryotic cells, we have chosen this virus for our initial experiments. Our hope is to construct an active DHFR gene which can be packaged into SV40 coat proteins providing a hybrid virus which can be used to infect cells thereby introducing the DHFR gene.

Preliminary DNA transformation experiments in our and other laboratories have yet failed to define the required DNA sequences surrounding a globin gene which result in a high level of expression when the gene is introduced into erythroid or other cell. An alternative strategy involves the use of viral sequences to facilitate either amplification or a high rate of transcription of the globin gene. Our initial plan again calls for use of SV40 signals but we also plan, if necessary, to use defined promoters from other viruses, specifically human adenovirus.

Methods:

1. Construction of potentially functional DHFR genes: All constructions are performed by use of the plasmid vector, pBR322, to allow cloning of constructs into E. coli. Restriction endonuclease fragments derived from SV40 or cloned sheep or human globin genes, are put in what is hoped to be an appropriate position with respect to the DHFR enzyme coding sequences to provide a promoter, RNA splice signals, and a polyadenylation-transcription termination complex. Individual fragments are inserted successively into a recombinant plasmid by ligation and then transformation into E. coli until the desired hybrid construct is obtained.

2. Construction of hybrid SV40 viruses: The principle for these constructions is the same as that outlined above except that the initial recombinant plasmid contains a deletion mutant of SV40. The construction of the hybrid virus already completed utilized a plasmid containing the SV40 fragment defined by Bam HI (0.14) and Hind III (0.94) as the starting material. Into this, at the Hind III site (0.94) and the plasmid Eco RI site was inserted a Hind III-Eco RI fragment containing the DHFR gene. Recovery of the closed circular hybrid viral DNA from the resulting recombinant plasmid was possible by cleavage at the Bam HI site of SV40 (0.14) and at a Bgl II site just 3' to the mRNA coding sequence for DHFR. Because these enzymes produce complementary ends, ligation generates a circular, potentially infectious DNA molecule.

3. Lytic infection of monkey kidney cells: These experiments have been performed in collaboration with Dr. Dean Hamer of the Recombinant DNA unit of NIAID. Hybrid viral DNA is mixed with DNA from a temperature sensitive helper virus which is defective in the early region. The mixture is introduced into permissive monkey kidney cells. Following complete lysis of the cells, a viral stock is harvested which may be then used in high titer to infect permissive monkey kidney cells. After approximately 40 hours and before cell lysis occurs, the cells are harvested and used for DNA extraction or preparation of an extract appropriate for enzyme assay.

4. Transformation of tissue culture or bone marrow cells with DNA: DNA containing the gene or genes of interest is mixed with carrier Herring sperm DNA and precipitated by incubation in a solution containing calcium and phosphate. The precipitated DNA is added to cells which are then incubated at 37° for 4-24 hours. After the DNA precipitate is removed, the cells are either incubated in vitro or in the case of bone marrow cells, reinjected into lethally irradiated mice. Selection for transformants either in vivo or in vitro is by challenge with methotrexate.

5. Microinjection of DNA into tissue culture cells: These experiments are performed in collaboration with Lillian Killos and W.F. Anderson of the Laboratory of Molecular Hemotology using techniques outlined in the annual report of that laboratory. DNA containing the gene or genes of interest is injected directly into the nuclei of various cells including monkey kidney cells, mouse L cells and mouse erythroleukemia cells. Transformants are selected by incubation in media containing methotrexate.

6. Characterization of transformed cells: DNA from transformed cells is analyzed by restriction endonuclease digestion, gel electrophoresis, and Southern blotting. RNA is annealed in solution to probes specific for DHFR or globin mRNA sequences to allow their quantitation. Gel electrophoresis and Northern blot analysis of RNA is used to define the size of specific RNA species. An assay for DHFR quantitates conversion of $[^3H]$dihydrofolate to $[^3H]$tetrahydrofolate providing a measure of enzyme activity.

Results :

1. Construction of potentially functional DHFR genes: Three constructs have already been completed.

a. DHFR gene has been inserted into the late region of SV40. This recombinant plasmid contains the entire early region of SV40, the SV40 origin of DNA replication, the late region promoter and splice signals, the DHFR mRNA coding sequences, and the late region polyadenylation-transcription termination complex. It serves as a source for the SV40 hybrid viral DNA containing the DHFR gene in the late region.

b. Constructed in pBR322 is a gene which contains the following components: the SV40 origin of DNA replication, the SV40 early region promoter, the DHFR mRNA coding sequences, the human β globin gene large intron with its RNA splice signals, and the sheep γ gene polyadenylation-transcription termination complex.

c. Assembled in the plasmid pBR322 are the following fragments: the SV40 origin of replication and early region promoter, the DHFR mRNA coding sequence, the SV40 t-antigen splice signal, and the SV40 late region polyadenylation-transcription termination complex.

2. Formation of a hybrid SV40 virus containing the DHFR gene in the late region: The recombinant plasmid described above (1a) was cleaved with Bam HI and Bgl II and the 5 kb linear hybrid viral DNA isolated. It was ligated into a circular form and used to co-infect monkey kidney cells along with DNA from a temperature sensitive virus with a defect in the early region. A viral stock was recovered. During lytic infection of monkey kidney cells, DNA was isolated and the presence of the SV40 hybrid virus containing the DHFR gene was confirmed by restriction endonuclease digestion and Southern blot analysis. RNA extracted from these cells during the late phase of viral infection was found to contain two RNA species which included DHFR coding sequences. The first was approximately 19 S while the second was approximately 12 S. The ratio of small to large mRNA species was only 1 to 10. The activity of DHFR in the cells infected with the hybrid virus was approximately 6 times greater than in control cells and 3.5 times greater than in cells infected with wild type SV40. The only modest increase in DHFR activity in the cells infected with the hybrid virus may be due to the fact that the predominant splice yields an RNA product with translation initiation codons located 5' to the initiation codon for the DHFR enzyme.

Significance to Biomedical Research and the Institute Program:

Many serious human diseases arise because of monogenetic mutations affecting a structural or enzymatic component within cells. Two of these in which we are particularly interested are sickle cell anemia and homozygous β-thalassemia. The goal of achieving genetic therapy for these conditions seems realistic given our current level of knowledge. In the course of pursuing this objective, additional knowledge will be gained regarding the function of transcriptional regulatory signals in eukaryotic cells.

Proposed Course of the Project:

Our immediate plans are to test the already constructed recombinants with respect to their potential for conferring methotrexate resistance onto tissue culture cells. Cells will be infected with virus, transformed with calcium phosphate precipitated DNA, and/or microinjected directly. If resistant clones of cells are obtained, these will be characterized as to their content of the recombinant DNA molecules, their level of expression of the DHFR gene, and the nature of the RNA products. Successful transformation of cells *in vitro* will be followed by attempts to introduce the methotrexate resistance marker into hematopoietic stem cells which will be reinjected into lethally irradiated recipient mice. If necessary, additonal constructs will be made which incorporate other replication and transcription signals if the current constructs do not yield a highly functional DHFR gene. Once a functional gene is identified, globin genes either in their native state or in a modified form will be linked to the active DHFR gene and used to transform erythroid cells. These will include mouse erythroleukemia cells in vitro and hematopoietic stem cells which will be studied in their in vitro environment.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 02308-01

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of DNA Sequences Surrounding the Human β-like Globin Genes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Kaufman	Research Hematologist	CHB NHLBI
	J. Adams	Research Associate	CHB NHLBI
OTHER:	P. Kretschmer	Visiting Expert	LMH NHLBI
	M. Harrison	Research Assistant	CHB NHLBI
	W. F. Anderson	Lab. Chief	LMH NHLBI
	A. W. Nienhuis	Branch Chief	CHB NHLBI

COOPERATING UNITS (if any)

Laboratory of Molecular Hematology, NHLBI

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Md. 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.0	0.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A library of recombinant bacteriophage containing the human genome was screened with a probe specific for globin gene sequences. Recombinants that contained either the ε, the two γ, or the 3' half of the β globin gene along with extensive flanking DNA were identified. The inserted DNA fragments within the clones containing the ε and the two γ genes were found to overlap; thus the linkage of these genes was established. The ε gene is 14 kilobases (kb) 5' to the $^{G}\gamma$ gene. Heteroduplexes formed between two recombinants containing either the two γ genes or the δ and β genes were examined for homology by electron microscopy. Heteroduplex formation was observed only between the coding sequences of the respective globin genes; the flanking sequences had completely diverged. Moderately repetitive DNA sequences were found at several locations throughout the β-like globin gene cluster. An unusually long repeated DNA sequence was found 3' to the β globin gene. Its length, determined by electron microscopy of heteroduplexes formed between recombinants containing different copies of the repeat, was found to be 6.4 kb. Approximately 3000-5000 copies of this repeat are present in the human genome; thus, this family of moderately repetitive DNA sequences represents approximately 0.5-1.0% of the total human genome.

Objectives:

The human ε,γ , and β globin genes are expressed sequently throughout ontogenic development resulting in a series of finely regulated switches in the major hemoglobin found in circulating red cells. Definition of the mechanism of these switches in hemoglobin phenotype has been pursued with a goal of using this as a model for understanding gene regulation in eukaryotic cells in general. Also specifically the switch from fetal (Hb F = $\alpha_2\beta_2$) during the perinatal period results in the appearance of the phenotypes characteristic of severe β-thalassemia and sickle cell anemia in those individuals who have the abnormal β globin genes responsible for these conditions. Thus, reversal of the fetal to adult switch in such individuals could be helpful in their treatment.

Molecular cloning recombinant DNA technology provides a means to obtain specific DNA fragments in pure form allowing detailed characterization of particular regions of the human genome. Because of our interest in the problem of hemoglobin switching, we have chosen to focus on the human ε-γ-β- globin gene cluster. Each of these genes is approximately 1600 base pairs (bp) in length but the entire gene cluster is dispersed over at least 46 kb of DNA. It has been postulated that DNA which does not code for protein must contain sequences involved in gene regulation, chromatin folding, or other poorly understood but potentially important functions: Our objective is to characterize the structural features of the DNA sequences surrounding the human β-like globin gene cluster thereby hoping to clarify their functional roles.

Methods

1. Identification of recombinant bacteriophage containing human globin gene sequences: A library of the human genome cloned as 15-20 kb fragments into *E. coli* using the bacteriophage vector, Charon 4A, was screened with a probe specific for β-like globin gene sequences. Plaques which gave a positive signal were re-plated and a secondary screen performed. Twice purified positive plaques were expanded. DNA was extracted, and specific fragments of particular interest were subcloned into *E. coli* using the plasmid vector, pBR322.

2. Restriction endonuclease mapping: Total human genomic DNA or DNA from specific recombinant bacteriophage was purified, digested with restriction endonucleases, and resolved by electrophoresis through agarose gels. The DNA fragments were visualized by staining with ethidium bromide and subsequently were transferred to nitrocellulose filter paper by the Southern blotting technique. Incubation of the nitrocellulose paper with radioactive probes. specific for globin gene or other sequences allowed fragments containing these sequences to be defined. By this general approach, restriction endonuclease maps of the genomic regions contained in specific recombinants have been constructed.

3. Analysis of DNA heteroduplexes by electron microscopy: DNA from any two specific recombinants whose inserted DNA fragments contained sequences which were potentially homologous to one another, was allowed to anneal under defined conditions. Formation of heteroduplexes was detected after approximate shadowing, by scanning grids with the electron microscope. Photographs of identified heteroduplexes were studies to define the lengh and location of homologous DNA in the two recombinants.

4. Determination of repetition frequency and sequence homology of specific DNA fragments: Specific cloned DNA fragments were rendered radioactive by a nick-translation reaction and used as probes in solution hybridization reactions with genomic DNA or DNA from specific recombinants. The rate of annealing provided a measure of the repetition frequency of the probe sequences while the thermal stability of formed duplexes provided a measure of the degree of sequence homology.

Major Findings

1. Linkage of the human ε and $^{G}\gamma$ genes: Two recombinants, one containing the human ε gene and the second, the two γ genes, were found to contain two Eco RI fragments of identical length. Other restriction endonuclease sites were identical within the two Eco RI fragments from the two recombinants. Furthermore, one of the two Eco RI fragments from the clone containing the ε globin gene was found to hybridize, in a Southern blot reaction, to the corresponding Eco RI fragment in the clone containing the γ globin genes. Thus, the linkage of the ε to the $^{G}\gamma$ gene was established; the ε gene is approximately 14 kb 5' to the first of the two γ genes.

2. Dishomology in the sequences flanking the γ and β globin genes: DNA heteroduplexes formed between two recombinants containing either the two γ or the δ and β globin genes were examined for regions of homology with the electron microscope. The coding sequences of the $^{A}\gamma$ and β genes formed duplexes as did a portion of the coding sequences of the $^{G}\gamma$ and δ globin genes. No other evidence of homology was found in the approximately 12 kb of DNA examined in these two clones. Thus extensive sequence divergence has occurred in the flanking regions of these globin genes.

3. Characterization of a long moderately repetitive DNA sequence found 3' to the human β globin gene: One recombinant isolated from our human genomic DNA library was found to contain the 3' half of the β globin gene and 17 kb of DNA located in the downstream or 3' direction. To characterize this region of the genome, we isolated specific restriction endonuclease fragments from this recombinant to be used as probes in Southern blot analysis against genomic DNA. From these experiments we learned that moderately repetitive DNA sequences were found 3' to the β globin gene. One fragment was used as a probe to screen our human DNA fragment library in order to obtain additional copies of this moderately repetitive DNA family. By solution hybridization experiments, examination of heteroduplexes formed between recombinants containing copies of this moderately repetitive DNA family, and by Southern blotting experiments the following information was obtained. The copy located 3' to the β globin gene is 6.4 kb long; other members of the family are likely to be of a similar length. There are approximately 3000-5000 members of this moderately repetitive DNA family within the human genome. Therefore, this family accounts for approximately 0.5-1.0% of total human DNA. Although some sequence differences among individual copies of the family are evident from restriction endonuclease mapping of individual clones, the thermal stability of duplexes formed between one half of one copy and human genomic DNA suggest only about 10-15% divergence within the family. Characterization of the internal structure of this moderately repetitive DNA family suggest a long linear repeat rather than shorter tandemly arrayed repetitive sequences. The most 3'-1 kb of the copy located downstram from the β globin gene is repeated in an inverted orientation another 5 kb downstream; the intervening DNA is unique or repeated at a low frequency in the human genome.

Significance to Biomedical Research and the Institute Program

The mechanism of gene regulation and other functions in which non-coding DNA sequences may be involved in the cells of higher organisms are central problems in modern molecular genetics. Our goals are to contribute to the elucidation of these mechanisms while our focus on the human β-like globin gene cluster reflects our interest in the human genetic disease, β-thalassemia and sickle cell anemia and the general problem of hemoglobin switching. The long term objective is to obtain insights which may permit the stimulation of fetal hemoglobin production in patients in whom this would be of potential therapeutic benefit.

Proposed Course of the Project

During the next several months we hope to initiate experiments which will provide us a transcriptional map of the human β-like globin gene region. Specifically we are interested in knowing the location of other repetitive DNA sequences and whether or not they are transcribed into cellular RNA. Also, the presence or absence of other structural genes within this genomic region is a question which must be resolved. Further characterization of the long moderately repetitive DNA family is planned. Specifically we hope to learn whether other copies of the family neighbor genes which are expressed in human erythroid cells. Furthermore, we hope to learn whether all or any portion of individual copies of this repetitive DNA family are expressed in cellular RNA. The appropriate experiments to determine whether analogous repetitive DNA families are found in other species, specifically higher primates, are planned. Finally, we hope to learn whether this long repetitive DNA sequence will facilitate transformation of human cells with DNA fragments containing this sequence and furthermore, if such transformation is obtained whether the repetitive DNA will facilitate integration of the transforming fragments into chromosomal DNA.

Publications

1. Kaufman, R.E., Kretschmer, P.J., Adams, J.W., Coon, H.C., Anderson, W.F., and Nienhuis, A.W. Cloning and characterization of DNA sequences surrounding the human γ, δ, and β globin genes. Proc. Natl. Acad. Sci. USA. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-02309-01 CHB

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Use of Hybridoma Technology in the Study of Erythroid Differentiation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Neal S. Young	Visiting Expert	CHB NHLBI
Other:	Lilly Lee	Biologist	CHB NHLBI
	Sheam-Pey H. Chen	Fogarty Scholar	CHB NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinical Hematology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 0.75	PROFESSIONAL: 0.75	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The specific, monoclonal antibodies produced by hybridoma technology may have important application to the study of erythroid differentiation. Using the techniques developed by Kohler and Milstein, we have applied this methodology in three broad areas to produce antibodies to interesting antigens: 1) hemoglobins A and F, antigens available in large quantity and high purity and for which antibodies have obvious applications in the measurement of specific hemoglobin types in human disease and in vitro culture; 2) erythropoietin and other growth factors, antigens which are available in very limited quantity and impure form for which specific antibodies may provide an important means of purification; and 3) antibodies to hematopoietic stem cells, especially the erythroid burst forming unit, the earliest human cell which is readily cultured in vitro. The inability to purify bone marrow stem cells to any degree of homogeneity is the major obstacle in the study of hematopoietic differentiation.

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PHS-6040
(Rev. 10-76)

Hybridoma technology has provided a method for the production of large quantities of monoclonal antibodies of restricted and often unusual specificity, a goal difficult and often impossible to realize with conventional immunological techniques which employ subtraction of unwanted specificities from whole antisera. Hybridomas are the cell fusion products, resulting from polyethylene glycol treatment of spleen cells from an immunized mouse and an immortal murine myeloma cell line. In selective media, the fused hybridoma cells may be cloned and the synthetic products of the cloned cells assayed in order to select cell lines producing antibody of desired specificity. Cloned cell lines are capable of producing microgram quantities of antibody in tissue culture flasks and milligram quantities when injected into animals as ascites producing tumors. Selection of specificity therefore occurs at the level of the individual antibody producting cell and not by manipulation of animal serum. In work with cell membrane antigens, hybridoma cells have been isolated which produce hybridoma antibodies which recognize "minor" specificities on the cell surface which are of major biological interests, for example, thymocyte sub-class antigenic markers, differentiation antigens, histocompatability antigens, and malignancy markers have been isolated by selection of appropriate clones, despite their apparent absence on analysis of whole serum. Second, hybridoma technology has major application in theory to the isolation and purification of immunogenic molecules, either on the cell surface or in solution, which are available only partially purified and in limited quantity, providing that a suitable assay for specific antibody to the molecule is developed. Finally, hybridoma cells and their antibody products are of intrinsic interest to immunologists in studies of antibody-antigen binding sites and the character of antigenic determinants which confer specific biochemical properties on proteins.

I. Hemoglobin

Objectives:

In our laboratory, previous experience with the conventional immunology of hemoglobins suggested the usefulness of this antigen to define the optimal conditions obtaining hybridomas, namely, animal immunization, cell fusion, and clonal growth. A large number of Balb C strain of mice have been immunized with chromatographically purified hemoglobins A and F, 200 μg day 1, 100 μg day 14, both given intraperitonealy in Freund's adjuvant, and 50 μg hemoglobin intravenously day 28 or after. Animals are sacrificed 3 days following the final intravenous innoculation. Although peak antibody levels to hemoglobin develop 12-14 days following boost with antigen and Freund's adjuvant, for purposes of hybridoma production, an intravenous injection 3-4 days prior to sacrifice appears to be an absolute requirement.

Methods:

NS-1, Sp-2, and P3X63.AG8 myeloma cell lines have been employed for fusion. All the myeloma cell lines are $HPRT^-$, but vary in their secretion of antibody molecules: P3X63, which has been employed in most of the published hybridoma work, secretes its own heavy and light chain, NS-1 secretes a light chain, and Sp-2 produces no myeloma antibody. NS-1 cells have proven to be most suitable for the majority of our studies, as exposure to 50% polyethylene glycol for 1-2 minutes results in approximately 400 clones/immunized spleen, with a high frequency of positives to hemoglobin (10-40%). Sp-2 fusions result in fewer clones (100/spleen), but dependent on the immunization schedule, a large proportion of these may be reactive to the injected antigen.

Screening for hemoglobin binding is performed in a plate assay. Hemoglobin is allowed to adhere to microtiter plates, supernatant or sera specimens are added to each well, and following washing the binding of radioionated protein A or anti-mouse immunoglobulin is measured in a gamma counter.

Results:

After establishing optimal fusion and growth conditions, a large number of clones which produce antibody to hemoglobins have been isolated. Using the immunization schedule outlined above, all antibodies, whether isolated from hemoglobin A or hemoglobin F injected mice, have been equally reactive with the two hemoglobins, despite the major differences in the amino acid sequence of these molecules.

Proposed Course of Project:

The hybridomas which produce antibody reactive to hemoglobins A and F have been expanded. Specific antibodies to hemoglobins A and F are of intrinsic immunologic interest and required for quantitative assay of hemoglobins in erythroid culture, human bone marrow, and peripheral blood. Immunologic techniques which manipulate the antibody response of the injected mice to antigens may result in production of hemoglobin antibodies of greater specifity. For example, spleens from hemoglobin F injected animals have been injected into irradiated animals hyperimmunized to Hb A; this sublethal irradiation destroys the recipient's antibody producing cells. At the time of injection of the hemoglobin F spleen cells, Hb F is injected intravenously. This experimental approach is predicated on the ability of high levels of antibody to specifically suppress the proliferation of clones synthesizing that antibody.

II. Erythropoietin

Objectives:

Human erythropoietin has been purified to homogeneity (70,000 units/mg) by conventional and very tedious biochemical techniques. A specific antibody to this molecule would allow for its affinity purification. It would also serve as a marker for the presence of erythropoietin and the experimental definition of its mechanism plus site of action, target cell specifity, physiology and pharmacology. Crude antiserum have been raised in other laboratories to impure erythropoietin which are capable of neutralizing the action of this effector, but these have proven inadequate for biochemical study. We have attempted to obtain monoclonal antibodies using hybridomas by immunizing Balb C mice with either commercially obtained sheep erythropoietin, which has been further purified by absorption to wheat germ agglutinin lectin Sepharose, or partially purified human erythropoietin, obtained from the Blood Resources Branch, NHLBI. Suitable assay methods for the detection of antibody erythropoietin have been developed.

Methods:

Balb C mice 4-6 weeks old have been immunized with 100-200 units sheep or human erythropoietin in Freund's adjuvant x 2 at 2 week intervals and by intravenous tail vein injection 3 days prior to sacrifice. The spleen cells from these animals have been fused with polyethylene glycol to a variety of myeloma cell lines, including P3X63.AG8, NS-1, and Sp-2 cells. Parent antisera and the supernatants obtained from microtiter wells containing the cloned hybridoma cells have been assayed employing a variety of methods:

A. Direct binding to highly purified erythropoietin prepared by Dr. E. Goldwasser and radioionated by us to high specific activity. Serum or supernatants are absorbed to anti-mouse IgG or protein A liganded to Sepharose, which allows separation of bound and free erythropoietin as in a conventional radioimmunoassay.

B. Inhibition of serum or supernatant of growth of erythroid cell colonies, specifically fetal liver cells in the presence of .1-1 unit/ml erythropoietin.

C. Indirect inhibition of erythroid cell growth. In these assays, hybridoma supernatants or serum are absorbed to plastic plates coated with protein A or anti-mouse IgG, erythropoietin is added to the dry plates and after suitable incubation, removed and assayed for ability to support erythroid cell growth.

D. Complement lysis of erythroid progenitors. Test supernatant or sera are added to erythropoietin saturated fetal liver cells. Further addition of anti-mouse IgG and complement results in lysis of these cells as detected by colony formation.

Results:

Several animals hyperimmunized with sheep erythropoietin died of anaphylaxis following intravenous injection. Because of limited quantities of human erythropoietin, two animals have been fully immunized with a total of 500 units each of human erythropoietin and fused with NS-1 cells. The serum of these animals did not inhibit in direct or indirect assay growth of murine fetal liver cells. There was evidence of only marginal binding, of their serum to ^{125}I- erythropoietin, compared to controls. The sera did lyse fetal erythroid progenitors in the presence of anti-mouse IgG and complement when compared to control serum raised against unrelated molecules. A total of approximately 700 hybridoma clones have been assayed using direct inhibition

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of fetal liver cell growth and binding to radioactively labelled erythropoietin as described above. No clones were inhibitory to erythroid cell growth under the conditions employed. A single clone bound ^{125}I- erythropoietin and with some difficulty has been expanded.

Proposed Course of Project:

During the last six months, it has become apparent that, in contrast to preparation of antibodies to cellular membrane antigens, the preparation of antibodies to soluble antigens, using the hybridoma technology, even when these are highly purified, is a formidable task. Many of the antigens used have been hormones and these may be inherently less immunogenic than cellular proteins. Because of their soluble nature, they may be processed differently in the immunized mouse, for example, cleared more rapidly from the circulation. There are major variations among mouse strains and within individual mice in responsiveness to antigens, presumably determined by immune response genes. The quantity of soluble antigens injected may have to be large - on the order of 1 mg/animal as total dose - to insure the production by the hybridomas of specific antibodies. This would require approximately 70,000 units of erythropoietin per animal for immunization alone, a quantity which our laboratory is not prepared to purify and which is unavailable from other sources. As an alternative to this investment of very large quantities of purified erythropoietin is the development of techniques which may enhance the immunogenicity of erythropoietin. As an example of the latter, we have prepared syngeneic erythroblasts coated with sheep erythropoietin and immunized several different mouse strains with these fixed cells. This method presents the foreign antigen on the insoluble cell surface and may enhance its immunogenicity.

III. K562 Leukemia Cells

Objectives:

The inability to purify the small number of stem cells present in the bone marrow in large quantity and in pure state is a major obstacle in studies of erythropoiesis. Production of antibody which is specific to a stem cell membrane antigen would offer a method for identifying and purifying this minor population of bone marrow cells. As isolation of these cells for immunization purposes as well as screening is difficult, as a first approach we have employed the K562 leukemia cell line as immunogen. K562 cells are derived from the ascitic fluid of a patient with chronic myelogenous leukemia; however, these cells have the ability to synthesize embryonic and fetal hemoglobins and alpha globin in their basal state and to increase globin chain synthesis and globin mRNA following induction with hemin, sodium butyrate, and other chemicals. K562 cell membranes contain glycophorin and other red cell membrane components. Sharing these properties with human erythroid cells but in other respects a primitive blast cell, it seems likely that the K562 cells may also share antigenic determinants with erythroid stem cells. Practically, K562 cells are easy to maintain in long term culture and readily available for assay purposes.

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Methods and Results:

Balb C mice have been immunized with 1-2 x 10^7 K562 cells in Freund's adjuvant at 2 week intervals and with K562 intravenously 3-4 days prior to sacrifice. Fusions have been performed with NS-1 and Sp-2 myeloma cells. It is important to irradiate the K562 cells prior to immunization as they may seed the spleen and subsequently overgrow the clonal cultures. The hybridoma supernatants are assayed for binding to K562 cells or peripheral blood lymphocytes which have been glutaraldehyde fixed and sedimented to the bottom of microtiter wells. Radioionated protein A has been used as a marker of immunoglobulin presence on cell surfaces. Employing NS-1 myeloma cells, a total of 348 clones were grown in 672 wells. Eighteen percent of supernatants showed binding activity greater than one standard deviation and 7% showed binding activity greater than 2 standard deviation above the mean of radioactivity bound to K562 cells. 7 clones (2%) demonstrated greater than 2 fold higher binding to K562 cells compared to peripheral blood lymphocytes. These clones are being expanded in culture for eventual injection as ascites producing tumor in Balb C recipients. Anti-K562 antisera bind K562 cells and in addition show strong fluorescence against K562 cells, some normal bone marrow cells, but not human erythrocytes. In a hybridization performed with Sp-2 cells, 71 single clones resulted from 576 wells plated. Of these clones, 6 were reactive to K562 cells, and 4 demonstrated significantly higher binding to K562 cells compared to peripheral blood lymphocytes.

Proposed Course of Project:

Following expansion and production of larger quantities of antibody, the hybridoma immunoglobulins will be assayed for binding to bone marrow cells from a variety of patient disorders using immunofluorescence for detection. Specifically sought will be high binding to erythroid compared to myeloid bone marrows and selective binding to primitive rather than mature erythroid cells by morphologic criteria. Second, the effect of these antibodies in combination on erythroid and myeloid progenitors in culture will be assayed. Eventually, fluorescent cell sorting will be used in an attempt to separate stem cells from total bone marrow using these antibodies.

In addition, normal bone marrow cells and peripheral blood lymphocytes will be used as primary immunogens. Peripheral blood lymphocytes will be obtained in large number using the automated cell separator. The peripheral blood lymphocytes will be depleted of T and B cells, leaving the less than 5% of the total mononuclear cells which are known to contain the stem cell compartment. The frequency of erythroid progenitors as measured by the BFU-E assay in this preparation should be 1/500-1000 cells. Also, bone marrow obtained from patients with dyserythropoietic anemias in which there is a high proportion of early erythroid cells will be employed as immunogen, anticipating that these bone marrow cells may have an abnormal expression of early stem cell antigens.

Isolation of antibodies to erythroid stem cell antigens would permit purification and study of pure populations of these cells in culture, specific viral transformation of the selected population, and assessment of interaction with other well defined populations of cells, such as T-lymphocytes. It is also potentially useful in bone marrow transplantation and in the definition of a variety of anemic states.

ANNUAL REPORT OF THE
LABORATORY OF EXPERIMENTAL ATHEROSCLEROSIS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1979 through September 30, 1980

The morbidity and mortality of atherosclerosis is caused by local overgrowths of the intimal lining of arteries. These overgrowths or "atheromata" are spatially discrete and tend to have a characteristic topographic distribution in a given species. Histologically these intimal thickenings are characterized by cellular hyperplasia, macrophage infiltration, connective tissue proliferation, and increased lipid deposition, especially cholesterol and cholesteryl ester. Although the role of the cellular proliferation in these lesions has received considerable attention recently, there is no evidence that proliferative changes in the absence of lipid deposition contribute to the morbidity of this disease. Accordingly, our efforts have focused on the events leading to intimal lipid deposition since this occurrence appears to be most closely correlated with the lethal consequences of this disease. Our major activities have been directed toward development of 1) topographic analytic techniques for study of the spatial patterns of the disease, 2) methodology to study the transport processes related to intimal lipid deposition, 3) definition of the nature and location of the barriers to transarterial lipid transport, and 4) further study of the altered lipoprotein metabolism associated with atherogenesis.

Topography. The topographic distribution of atherosclerosis is studied from opened arterial specimens that have been stained with Sudan IV. Regions of increased intimal lipid deposition appear as sudanophilic patches on the opened arterial tree. The sudanophilic pattern that appears early in the disease is characterized as simple fatty streaking; however, in a matter of months certain areas of this pattern evolve into typical atheromatous plaques. This is particularly true of the coronary arteries, lower abdominal aorta, and larger peripheral arteries of primates and swine. The predictability of these patterns at given locations suggests that appropriate statistical approaches using modern image processing techniques will permit study of the sequence of local events leading to the complicated atheromatous plaque.

Therefore we have pursued development of automated image processing techniques so that we can establish correlations of local structural, biochemical, and mechanical events with the evolution of the atheroma. This approach is made possible by standardized necropsy and formatting procedures that we have developed. Vessels are opened and pinned flat in a standard format on which it is possible to identify from 10-50 invarient anatomical landmarks to be used as fiducial points. The x-y coordinates of each are taken in sets of three to calculate the six coefficients necessary for a linear transformation of the data contained within the triangle bounded by the three fiducial points onto the corresponding triangle of a standardized coordinate system. The necessary scanning procedures (using microdensitometry from photographic transparencies or using television from the opened specimens at necropsy) have been worked out with collaborators from the Division of Computer Research and Technology and the Biomedical and Instrumentation Branch. The necessary image processing software and statistical analytic

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algorithms have been developed and checked with pilot studies. This interdisciplinary effort will represent a major advance in our ability to carry out the indicated topographic correlations.

Transport. As described in previous annual reports, we have demonstrated that the principal barrier to the flux of plasma substances into the arterial wall is the endothelial surface. We have demonstrated that the permeability and structural characteristics of the endothelial surface are exquisitely sensitive to the intensity of shear stress created by the adjacent blood flow and, of even greater interest, that the endothelial surface appears to compensate both structurally and functionally for altered stress patterns with time, provided the stress is unidirectional. In vivo study of the mechanisms by which this surface responds to changing directions of stress has been technically very difficult. Accordingly, we have put considerable effort this year into developing an in vitro system in which it is possible to study acute changes of endothelial permeability, not only in response to changes in the magnitude but also to changes in the direction of the imposed shear stress. These acute studies have been carried out using the canine and the porcine descending thoracic aorta and have provided data consistent with prior in vivo work. Efforts are currently directed toward better metabolic support for the tissue so that we can examine responses over longer time frames.

We have also continued to study the albumin-Evans blue dye (EBD) ligand system (using EBD-albumin and ^{125}I-albumin simultaneously) as a model for macromolecular ligand transport across arterial tissue. Background studies have been described in previous annual reports. This year we have made important advances in our understanding of detailed transvascular transport in arterial tissue, as well as in the development of new methodology for the discrete measurement of concentration distributions of protein and ligand across the arterial wall. The transport of albumin across the arterial wall is governed by two processes, diffusion and convection. In the absence of a pressure acting across the wall and in the absence of an endothelial surface, the uptake of radiolabeled albumin was shown to follow (to a first approximation) a "square-root-of-time law," indicating that the wall was behaving similar to a "homogeneous slab" model for diffusion. In the presence of a normal canine endothelial surface, the uptake was found to be more linear with time but greatly reduced. In a period of one hour, from five to ten times less albumin enters the normal wall than enters the "endothelial-injured" wall. These data are consistent with those mentioned earlier, indicating that the endothelial surface acts as a discrete but very large barrier to the transport of protein from the plasma phase to the arterial phase.

In vitro studies to examine the role of increased wall stretch on the transport processes have shown that the permeability of the endothelial surface is increased by increased stretch in the absence of an increased pressure. In contrast to this the transport processes in the intimal-medial portion of the system were shown to be virtually independent of the degree of wall stretch.

Other studies this year, designed to study the role of increased transmural pressure on transport processes in the canine and porcine aorta, showed that pressure itself can significantly increase the transport of labeled proteins across the deendothelialized intimal-medial preparation. For example, a transmural pressure of ∿100 mmHg increases the uptake of labeled albumin by approximately twofold.

A special technique was devised whereby the separate influence of diffusion and convection could be isolated. It was found that in the deendothelialized wall, convective transport was of the same order of magnitude as the steady-state diffusive transport. Similar studies for the intact, normally endothelialized artery suggest that increased pressure increases uptake little, if at all. These observations emphasize further the importance of the role played by the endothelial surface.

More information can be obtained regarding these processes by studying the discrete concentration distribution of a particular radiolabeled protein across the arterial tissue under experimentally imposed conditions of time, concentration, and manipulated physiologic and pharmacologic states. Until now, technology for this has been possible only under very special experimental circumstances. We have developed methodology so that these measurements can be done now in a variety of in vitro and in vivo experimental configurations and with a high degree of spatial resolution. To do this, quantitative autoradiographic techniques were developed in which the local concentration of silver in the developed autoradiograph is measured by scanning across the micrograph with an electron microprobe or microdensitometer. These experiments demonstrated that the relationship between the concentration of radiolabeled protein at any point in the wall is directly proportional to the adjacent developed silver in the microautoradiograph. The initial application of this new methodology has been to determine the distribution of radiolabeled albumin across deendothelialized aortic and iliac arterial walls in the transient and in the quasi steady state. The results of these studies have shown that the partition coefficient for radiolabeled albumin is nominally constant across the arterial wall (0.1-0.2) and that the coefficient of diffusion for albumin in arterial tissue is about 10^{-8} cm^2 sec^{-1} but tends to vary somewhat across the wall in relation to various histologic structures. When a transmural pressure of ∿100 mmHg is imposed on the system, the resulting concentration distribution differs significantly in shape and in magnitude from that of simple diffusion. The most noteable difference is the development of local tissue concentrations in excess of the diffusion equilibrium concentration indicating signficant macromolecular "sieving" by the tissue matrix. The significance of these studies is twofold: This new methodology provides an experimental tool that should be applicable to many other types of tissue systems for measurement of radiolabeled protein distributions of interest. Second, we can begin to study the parameters of macromolecular transport across the arterial wall in sufficient detail that, for the first time, it should become possible to model these processes by physical and mathematical laws. This should permit inferences regarding detailed distributions of various macromolecules, such as atherogenic precursors, across the arterial wall from relatively simple measurements of uptake. Finally, since this methodology provides access to detailed events within the wall, it

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opens the way to study of the ancillary metabolic events such as binding and efferent transport of cholesterol-rich metabolites out of the wall. Thus we have made significant progress toward developing the experimental and conceptual tools necessary for further definition of the nature of the unbalanced set of transport and metabolic processes associated with the intimal lipid deposition of atherosclerosis.

Lipoprotein and arterial metabolism. Studies have continued on dietary-induced atherosclerosis in dogs, miniature swine, and Patas monkeys. As reviewed in previous reports, the plasma lipoprotein patterns in these studies characteristically show an elevated LDL, BVLDL, and HDL_c. The increased levels of these proteins correlates with the rapidly developing appearance of atherosclerosis in these animals. Efforts this year have been directed toward determining whether any of the above lipoproteins are the direct result of chylomicron metabolism since their elevation appears to be dietarily induced.

Radioactive retinol- and cholesterol-labeled chylomicrons were obtained in donor animals from surgically constructed thoracic lymph duct fistulas. The labeled chylomicrons were injected intravenously into both normal and hypercholesterolemic recipients. It was found that the intravenously-administered labeled chylomicrons were rapidly ($T_{\frac{1}{2}} \approx 10$ min) cleared from the plasma compartment and that most of the administered radioactivity quickly appeared in the liver in both the normal and the hypercholesterolemic recipients. The failure of significant radioactivity to accumulate in the plasma and the rapid appearance of the radioactivity in the liver indicated that the labeled chylomicron remnants were not sequestered in the plasma compartment nor did they equilibrate with any of the other plasma pools of cholesterol (LDL, BVLDL, or HDL_c) prior to hepatic uptake. Accordingly, the hypercholesterolemia in dogs is not the result of chylomicron remnant accumulation in the plasma. The cholesterol-rich lipoproteins (BVLDL and LDL) are of hepatic origin and are not chylomicron remnants. In an effort to characterize this remarkably rapid removal of chylomicron remnants by the liver, chylomicrons will be injected into hepatectomized animals so that we may better observe the accumulation of the remnants. The plasma lipoproteins from these animals will be characterized and the labeled remnants isolated. These will then be reinjected into normal and hypercholesterolemic recipients to see if these are rapidly removed by the liver in accordance with the above observations and, if so, to evaluate their ability to compete with other lipoproteins for the hepatic remnant "receptor." These studies should provide new information regarding the sequence of events set in motion by cholesterol ingestion and which result finally in the appearance of those lipoproteins associated with accelerated atherogenesis.

Concluding Comment

We have made important advances in our understanding of dietary hyperlipoproteinemia. It appears that the "atherogenic lipoproteins" are not direct metabolites of intestinal chylomicrons, at least in the dog. We have acquired new insights into the processes associated with macromolecular transport in the arterial wall. The nature of these processes varies significantly depending upon the competence of the endothelial surface and the presence or absence of a transmural pressure. We have also developed new technology bringing us closer to our goal of studying the local physical and biochemical processes associated with atherogenesis in the living tissue system.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02812 11 EA

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Animal models for study of atherosclerosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
	G.W. Melchior	Investigator	EA NHLBI
	K. Kruth	Senior Investigator	EA NHLBI
Other:	J.E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	OD NHLBI
	J.F. Harwell	Head, Laboratory Animal Medicine	OD NHLBI
	T.L. Wolfle	Assistant Head, Carnivore Unit	VR DRS

COOPERATING UNITS (if any)

Veterinary Resources Branch, DRS; Section on Laboratory Animal Medicine, NHLBI; Comparative Medicine Unit, DRS

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Comparative Atherosclerosis and Arterial Metabolism Section

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study is to develop a dietary protocol and maintain metabolic conditions which are conducive to the development, in various animal species, of experimental atherosclerosis similar to human disease. This has been achieved in dogs, miniature swine, and Patas monkeys. For these species the source of dietary fat has been found to profoundly affect the type, distribution, and severity of the disease. Atherosclerosis produced by diets containing beef tallow is severe and is associated with arterial thrombosis and occlusive vascular disease.

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PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To determine the suitability of a variety of animals as models for studying human atherosclerosis.

Methods: The animal models which have been studied in varying detail are Patas monkeys, miniature swine, dogs, rabbits, and rats. The experimental conditions under which the pathologic processes in these animals can be made to resemble those in man have been detailed in previous project reports. Briefly, the disease can be induced in rabbits, swine, and monkeys by feeding diets high in cholesterol and fat (lard, beef tallow), whereas the disease can be induced in dogs and rats to a comparable extent only if hypothyroidism is also induced. In dogs, the dietary fats have included cottonseed oil, pork lard, beef tallow, safflower oil, and peanut oil.

Blood chemistries, including detailed lipoprotein studies, are monitored during the experimental period. Detailed lipid analyses of the platelets and erythrocytes from the animals on various diets are done by standard procedures. At termination each animal is examined in detail using the standardized necropsy procedures described previously. Topographic distribution and histologic characteristics of aortic, coronary, and peripheral arteries are compared to those of human atherosclerosis. The comparative human material is derived from young adults dying traumatic deaths, unselected hospital cases, and patients with documented types of hyperlipoproteinemia. A computerized coordinate system for plotting the topographic distribution of the atherosclerotic lesions has been developed.

Major Findings: The animal colony studies in progress for several years continue to provide the basis for our understanding of several key questions in the field of lipoprotein metabolism and atherosclerosis. One of the strengths of our program has been the ability to compare the response of atherosclerosis-resistant (dog and rat) species with susceptible (swine and monkey) species and to relate these findings to human disease. The approach has provided fundamental, basic information in several areas as follows:

1) Lipoprotein Metabolism. Certain consistent features of cholesterol-induced hyperlipoproteinemia in the various species have been described including the occurrence of a previously unrecognized class of plasma lipoproteins. The detailed understanding of the types of changes induced by cholesterol feeding in the animals (dogs, swine, rats, rabbits, and monkeys) has provided a background for studying the changes which occur with cholesterol feeding in man. A fundamental observation which is being actively studied in our laboratory is that the consumption of a cholesterol-rich diet by man, with or without an elevation of plasma cholesterol alters the plasma lipoproteins in ways which resemble the changes observed in the lower species. In the animal studies such changes are associated with accelerated atherosclerosis.

2) Regulation of Cholesterol Metabolism in Arterial Smooth Muscle Cells and Fibroblasts by Plasma Lipoproteins. A recent contribution of our laboratory, which was provided by the uniqueness of the cholesterol-induced lipoproteins of dogs and swine, has been the identification of the determinants responsible for lipoprotein binding to specific cell surface receptors. It is now established that the protein moieties of the lipoproteins are responsible for specific binding, that the B and arginine-rich (E) apoproteins are responsible for the receptor binding, and that arginyl residues of the proteins are functionally important in the lipoprotein recognition site. In addition, the regulation of the rate and extent of intracellular deposition of cholesteryl esters by arterial cells appears to be controlled by the type of lipoprotein which interacts with the receptor.

3) Role of Dietary Fat in Determining the Severity of Atherosclerosis and the Associated Complications of Thromboatherosclerosis. Dogs fed high-cholesterol diets containing either saturated or unsaturated fats develop two type of atherosclerosis, different in both distribution and severity. Diets containing saturated fat cause more severe atherosclerosis and a significant increase in the occurrence of thrombosis and the complications of thromboatherosclerosis (visceral organ infarction). Thromboatherosclerosis in dogs (and more recently in swine) on a high saturated fat-cholesterol diet has been correlated with platelet hypersensitivity to aggregation and release. These models provide for the first time an experimental approach by which to determine whether the role of the platelet in atherosclerosis is primary or secondary. Lipid analyses have revealed significant changes in the fatty acid composition of the platelets depending upon the type of diet. In addition, these well-characterized models provide the opportunity to determine experimentally the factors which alter the course of the disease process. The animal models provide the only approach to many of the questions which need to be explored if we are to understand the role of diet, trace elements and vitamins, plasma lipoprotein levels, and hemostatic function in the pathogenesis of atherosclerosis. Many of the advances we have made relate directly to observations made first in the animals and then extended to man.

Significance: The "atherosclerotic process" is, in fact, an ensemble of processes occurring at the cellular, physicochemical, biochemical, and biophysical level in the arterial intima. The purpose of this program is to identify as many of these fundamental processes as possible, establish which are relevant to the disease process in man, and study these in great detail in the animal model, wherein the pertinent variables can be measured or controlled with a rigor not possible in man.

A clear definition of the role of diet in the development of atherosclerosis in man is of utmost importance.

Proposed Course: The pursuit of the above objectives will continue with our NIH collaboration.

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PUBLICATIONS

Pitas, R.E., G.J. Nelson, R.M. Jaffe, R.W. Mahley. Effects of diets high in saturated fat and cholesterol on the lipid composition of canine platelets. Lipids. 14:469-477, 1979.

Mahley, R.W. Dietary fat, cholesterol, and accelerated atherosclerosis. In Atherosclerosis Reviews, edited by R. Paoletti, A.M. Gotto, Jr. Raven Press. New York. Vol. 5, 1-34, 1979.

Reitman, J.S. and R.W. Mahley. Yucatan miniature swine lipoproteins: Changes induced by cholesterol feeding. Biochim. Biophys. Acta. 575:446-457, 1979.

Mahley, R.W., D.K. Johnson, G.J. Pucak, and D.L. Fry. Atherosclerosis in the Erythrocebus patas, an Old World Monkey. Am. J. Path. 98:401-419, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02814 05 EA

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Topographic analyses of endothelial surface permeability and atherosclerosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
Other:	D.K. Johnson	Chief, Veterinary Medicine and Surgery Section	VR DRS
	J.E. Pierce	Chief, Section on Laboratory Animal Medicine and Surgery	OD NHLBI
	W. Barrett	Staff Fellow	CR CSL
	E. Hall	Electronics Engineer	BEI DRS
	G.J. Johnson	Biological Lab Technician	EA NHLBI
	R.C. Seabron	Biological Lab Technician	EA NHLBI

COOPERATING UNITS (if any)

Veterinary Resources Branch, DRS; Computer Systems Laboratory, DCRT; Biomedical Engineering and Instrumentation Branch, DRS

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS: 2.25	PROFESSIONAL: 1.25	OTHER: 1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To develop standardized necropsy and formating procedures to study topographic correlations in experimental atherosclerosis.

PROJECT DESCRIPTION

Objective: To develop standardized necropsy and formating procedures to study topographic correlations in experimental atherosclerosis.

Methods: A systematized necropsy procedure and standardized format was developed for arranging and fastening the opened arterial tree to reflective panels for optical scanning and analysis. This standardized format makes possible quantitative analysis of the topographic distribution of intimal Evans blue dye accumulation (a measure of the permeability of the wall to plasma substances) and of intimal lipid deposition along the arterial tree of experimental animals. This standardized format may be expressed in a normalized coordinate system for automatic optical scanning and computer pattern analysis or may be used directly for comparative visual analysis. This format permits careful, detailed comparisons of areas of increased arterial wall permeability with areas of intimal lipid deposition at identical sites from animal to animal.

Major Findings: Application of these techniques to a recently completed study in experimental atherosclerosis in swine demonstrated that the disease pattern occurs with an extraordinary congruency from animal to animal. It was also shown that the patterns of increased endothelial permeability in normal animals are very similar to the patterns of intimal lipid deposition that were characterized by very high permeability in normal animals, a characteristic atheromatous lesion occurred which, under the above standardized necropsy procedures, appeared as a tri-colored lesion. These lesions were characterized by relatively normal surrounding intimal surface (white), a highly permeable (blue) center bordered by an annular but otherwise typical atheromatous plaque (red). With time, these lesions were shown to form mature atheromatous plaques in the abdominal aorta; however, in the thoracic aorta and subclavian artery these appear to mature more slowly.

Significance: The extraordinary congruency of the disease patterns from animal to animal in a given species, as well as to the patterns of permeability in the normal animal of that species, represents a major finding of fundamental importance not only in linking the role of increased permeability to the subsequent development of this disease but also in permitting one to develop new strategies in experimental design to gain deeper insight into the associated atherogenic processes. The predictability of this process at given locations permits one to design studies to follow the sequence of events leading from the "earliest lesion" on through the complicated lethal atheromatous plaque. This capability was heretofore unavailable for lack of a standardized quantitative approach to the analyses of the disease topography and permeability topography. Moreover, the occurrence of the tricolor lesion provides the opportunity to study an apparent spectrum of atherogenic processes in a very discrete anatomical region.

This methodology will make possible an approach to a number of fundamental questions: Why do some regions of moderate endothelial permeability progress only to the fatty streak lesions whereas others go on to mature atherosclerotic lesions? Why do certain local regions of extraordinarily high permeability appear to be relatively immune to lipid deposition? What is the sequence of events leading from a fatty streak to a lethal atheromatous plaque? How are the patterns of streaking and plaquing influenced by hemodynamic alterations, hematologic manipulations, dietary triglycerides, and exercise?

Proposed Course: The progress of this large project has been slowed by several unanticipated problems related to the large data processing problems posed by this objective. Analysis has involved relatively detailed and sophisticated computer data processing techniques. We have finished the necessary pilot studies to establish the methodological and computational concepts. These are now being implemented with the necessary image capture and processing hardware and software. Initial applications will be to assess the influence of a variety of dietary and other factors on the evolution of this important disease process.

PUBLICATIONS

Lutz, R.J., J.N. Cannon, K.B. Bischoff, R.L. Dedrick, R.K. Stiles, and D.L. Fry. Shear stress patterns in a model canine artery: Their relationship to atherosclerosis. In Quantitative Cardiovascular Studies. Clinical and Research Applications of Engineering Principles, edited by N.H.C. Hwang, D.R. Gross, D.J. Patel. University Park Press. Baltimore. pp. 233-237, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02822 01 EA

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Dietary-induced hypercholesterolemia in dogs: chylomicron metabolism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: G.W. Melchior	Investigator	EA NHLBI
D.F. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI
D.K. Buckhold	Senior Veterinary Officer	OD NHLBI
R.W. Mahley	Gladstone Foundation Laboratories University of California (Formerly the Head, Comparative Atherosclerosis and Arterial Metabolism Section	EA NHLBI

COOPERATING UNITS (if any)

Section on Laboratory Animal Medicine

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.5	1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Thoracic duct lymph chylomicrons, doubly labeled in vivo with [1-^{3}H]retinol and [4-^{14}C]cholesterol were administered intravenously to both normal and hypercholesterolemic foxhounds to determine to what extent cholesteryl ester rich chylomicron remnants contribute to the dietary-induced hypercholesterolemia in these animals. The rapid removal of chylomicrons from the circulation and the subsequent recovery of the radioactivity in the livers of these animals indicated that none of the large plasma pools of cholesterol (B-VLDL, LDL, or HDL_C) represented chylomicron remnants accumulating in the plasma.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To estimate the contribution of cholesteryl ester rich lipoproteins of intestinal origin (chylomicrons and chylomicron remnants) to the dietary-induced hypercholesterolemia in foxhounds and identify lipoprotein families (B-VLDL or HDL_c, for example) that might represent chylomicron remnants.

Methods: Chylomicrons labeled in the lipid moiety with [1-^{3}H]retinol, [4-^{14}C]cholesterol, and/or [9,10-^{3}H]oleic acid were obtained from donor foxhounds in whom thoracic lymph duct fistulas had been created surgically. The labeled chylomicrons were injected intravenously into both normal and hypercholesterolemic recipients and their removal from the plasma and uptake by the liver was followed for several hours.

[1-^{3}H]retinol was chosen as a label because it has been shown by others to be esterified and incorporated into the lipid core of the chylomicron in the intestine and to remain with the chylomicron remnant after triglyceride removal in the periphery. Chylomicron remnants are normally rapidly removed from the circulation by the liver and the retinyl esters are either stored in the liver or returned to the circulation as free (nonesterified) retinol bound to a high density protein. Thus retinyl-ester labeled lipoproteins present in the plasma are presumed to be of intestinal origin.

Retinol is preferable to cholesterol as a chylomicron label because it is thought not to be recirculated by the liver in lipoproteins; and the plasma exchange of retinyl esters among the lipoproteins is very low, which is not the case for cholesteryl esters.

Major Findings: Retinol- and cholesterol-labeled chylomicrons administered intravenously were rapidly cleared from the plasma compartment and the radioactivity subsequently appeared in the livers of both normal and hypercholesterolemic recipients. The failure of significant radioactivity to accumulate in the plasma and the rate at which the radioactivity appeared in the liver indicated that the labeled chylomicron remnants did not equilibrate with any large plasma pools of cholesterol (LDL, B-VLDL, or HDL_c) prior to hepatic uptake.

Significant quantities of the retinol label (10-15%) were detected in the low density lipoproteins of both normal and hypercholesterolemic dogs after the intravenous administration of $S_f>400$ chylomicrons. This observation is contrary to previously published schemes for retinol metabolism and indicates that some chylomicrons are catabolized to LDL in the periphery, or that the liver recirculates retinyl esters in LDL.

Significance: It is agreed by most that the hypercholesterolemia produced in foxhounds is the result of dietary overloading, i.e., the cholesterol input from absorption exceeds the animal's capability to excrete cholesterol. The resulting imbalance is probably temporary, and a new steady state is eventually reached. It is not known whether the hypercholesterolemia results from the accumulation of dietary particles in the plasma compartment

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because of a saturated liver clearance mechanism or whether newly absorbed cholesterol is cleared from the plasma by the liver and recirculated in lipoproteins of hepatic origin. Our data indicate that the hypercholesterolemia in dogs is not the result of chylomicron remnants accumulating in the plasma and suggest that the cholesteryl ester rich lipoproteins (B-VLDL and LDL) are of hepatic origin.

Proposed Course: Chylomicron remnants are currently being produced in vivo by administering doubly labeled $S_f>400$ chylomicrons to hepatectomized dogs. The remnants so obtained will be completely characterized and then reinjected into normal and hypercholesterolemic recipients to a) evaluate their ability to compete with other lipoproteins for the hepatic receptor, and b) try to determine which component (e.g., which apoprotein) is responsible for recognition by the remnant receptor.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02823 01 EA

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cellular localizing factors in vascular cholesterol deposition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H.S. Kruth	Senior Investigator	EA NHLBI
D.L. Fry	Chief, Laboratory of Experimental Atherosclerosis	EA NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Experimental Atherosclerosis

SECTION
Vascular Physiology

INSTITUTE AND LOCATION
NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	.5	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Microscopic observation of atherosclerotic lesions reveals that both focal extracellular and selective intracellular lipid deposition occur. While some smooth muscle cells within atherosclerotic lesions contain substantial lipid, others are essentially lipid free. The reasons for the focal nature of extracellular lipid deposition and restricted accumulation of lipid by certain cells is not known. This project will assess the importance of cell injury and cell proliferation as localizing factors in the focal deposition of cholesterol within atherosclerotic lesions.

PROJECT DESCRIPTION

Objective: To investigate the role of cellular factors such as cell proliferation and cell injury in contributing to cholesterol deposition within atherosclerotic lesions.

Methods: Microhistochemical techniques including specific localization of cholesterol using the fluorescent probe filipin will be carried out on atheromatous vascular tissue. Techniques to detect cycling the injured cells will be developed. Use of cultured vascular cells will complement tissue studies.

Significance: An understanding of cellular factors which contribute to focal vascular cholesterol deposition may suggest future strategies for intervention in this pathologic process.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02824 01 EA

PERIOD COVERED

October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Use of flow cytometry in atherosclerosis research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: H.S. Kruth	Senior Investigator	EA NHLBI
Other: R.J. Romanoff	Computer Specialist	CR CSL
I. Levy	Electronics Engineer	CR CSL

COOPERATING UNITS (if any)

Division of Computer Research and Technology

LAB/BRANCH

Laboratory of Experimental Atherosclerosis

SECTION

Vascular Physiology

INSTITUTE AND LOCATION

NIH/NHLBI-DIR, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.25	.25	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is now recognized that endothelial cell injury is an important initiating event in atherogenesis. Endothelial injury and desquamation may be induced by either physical (e.g., hemodynamic injury) or biochemical induced injury (e.g., hypercholesterolemia, endotoxemia, or homocysteinemia).

A project will be initiated to assess the potential of using flow cytometry (allows rapid measurement of fluorescence of stained single cells flowing in suspension) to quantify circulating endothelial cells as an index of vascular injury. Additionally, flow cytometry will be evaluated as a means of analyzing cell suspensions prepared from atheromatous lesions.

PHS-6040
(Rev. 10-76)

PROJECT DESCRIPTION

Objective: To assess the potential of flow cytometry as a research tool in atherosclerosis research.

Methods: A fluorescence-activated cell sorter will be used to quantify selectively stained endothelial cells in peripheral blood samples from animals fed hypercholesterolemic diets. In addition, the feasibility of preparing single cell suspensions from atheromatous lesions for flow cytometric analysis will be determined.

Significance: The ability to quantify desquamated circulating endothelial cells in peripheral blood may provide a noninvasive measure of in vivo vascular injury. More generally, flow cytometry may provide a new research tool useful in atherosclerosis research.

ANNUAL REPORT OF THE
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1979 through September 30, 1980

Section on Experimental Therapeutics

The work of the Hypertension-Endocrine Branch has included studies of several of the major vasoactive systems: i.e., 1) renin-angiotensin-aldosterone, 2) sympathetic nervous system - catecholamines, and 3) kallikrein-kinin system. These studies were undertaken to delineate the pathogenesis of hypertension and to develop better forms of therapy for this disease.

A mother and her daughter with dexamethasone-suppressible aldosteronism were studied to help characterize this rare disorder that consists of hypertension, hyporeninemia and dexamethasone-suppressible aldosteronism. It is known that the disorder is familial with an autosomal dominant mode of inheritance which is not HLA linked. It is also known that treatment with dexamethasone corrects the aldosteronism and hypertension and restores the responsiveness of the renin-angiotensin system while treatment with adrenocorticotrophin (ACTH) produces a sustained increase in aldosterone production and blood pressure. But the cause of the ACTH-dependence of aldosterone production is uncertain. In the mother, dexamethasone treatment gave improvement by suppression of aldosterone while during spironolactone therapy or during pregnancy improvement was produced by antagonism of aldosterone. During the administration of ACTH, treatment with aminoglutethimide markedly decreased aldosterone but did not affect plasma cortisol or urinary 17-hydroxycorticosteroids. The decrease in aldosterone was associated with an increase in serum K^+ and a fall in blood pressure. Thus the responses to spironolactone, to pregnancy and to aminoglutethimide demonstrate the important role of sodium-retaining steroids from the zona glomerulosa, such as aldosterone, as mediators of the hypertension. Treatment of mother and daughter with ACTH produced a marked and sustained increase in aldosterone with a further increase in blood pressure. This observation that ACTH can further stimulate the overproduction of aldosterone that has been shown to be suppressible by dexamethasone indicates that ACTH is responsible for this overproduction and implies that conversion of deoxycorticosterone to aldosterone is also sustained and that the usual ACTH-induced decrease in 11-hydroxylation and 18-hydroxylation in zona glomerulosa cells does not occur. The mechanism by which ACTH normally inhibits these mitochondrial enzymes in zona glomerulosa cells and the reason that such an inhibition does not occur in dexamethasone-suppressible aldosteronism remain to be determined.

For several years there have been reports of renin-like activity (RLA) in blood vessels. However, these reports have been plagued by low levels of RLA and the presence of nonspecific acid proteases (NAP). We undertook an extensive study of the methodology and found that a mixture of EDTA, neomycin, and phenylmethylsulfonyl fluoride was needed to protect the RLA and the angiotensin I produced in the incubation from proteolytic digestion. In addition since nonspecific proteases such as the cathepsins can produce angiotensin I-immunoreactive material from renin substrate we had to completely separate the RLA from the NAP. This was accomplished by chromatography over a column of bovine hemoglobin coupled to Sepharose-4B. When sodium depleted rats were studied before nephrectomy and at two hours and 24 hours post-nephrectomy, the partially purified vascular RLA fell in parallel with the PRA. In animals studied 24 hours after bilateral nephrectomy and 15 hours after hemorrhage the vascular RLA was increased over that of controls which were nephrectomized and not hemorrhaged. Thus with our new method we could completely separate RLA from NAP and show two things: 1) After nephrectomy alone, the parallel fall of RLA and PRA suggests that RLA may arise from the plasma. 2) The marked increase in RLA after hemorrhage indicates that vascular RLA can be independent of PRA. These findings are of more than passing interest since they may suggest a site of action for angiotensin-converting enzyme inhibitors such as captopril which is a potent anti-hypertensive drug the action of which cannot be explained by other mechanisms.

Patients with Bartter's syndrome are resistant to the pressor effects of angiotensin II and norepinephrine. This increase in resistance to vasoconstrictors is associated with an overproduction of prostaglandins and is corrected by treatment with a prostaglandin synthetase inhibitor. We have studied 7 patients with Bartter's syndrome and have shown that the increase in pressor resistance to norepinephrine is associated with an increased excretion of norepinephrine in the urine and normal levels of epinephrine, metanephrine and VMA. Treatment with indomethacin decreased significantly the urinary excretion of norepinephrine, metanephrine and VMA. These results indicate that the increased rate of excretion of norepinephrine is associated with a normal rate of excretion of metabolites and is not due to a subnormal rate of metabolism of norepinephrine. The data suggest that the vascular resistance to norepinephrine is due to a prostaglandin dependent increase in sympathetic nerve activity.

It is often said that stress can raise blood pressure. Yet there is very little evidence to support this statement. We therefore decided to study cardiovascular, biochemical and psychological responses to a common form of stress, i.e., wisdom tooth extractions in young adults. We measured heart rate, blood pressure, cardiac output, plasma norepinephrine, epinephrine, growth hormone, cortisol, cholesterol, triglyceride, lipoprotein, pain and anxiety before, during, and after each of two sets of wisdom tooth extractions. Subjects were randomly assigned to receive either, neither, or both intravenous valium or epinephrine in the local anesthetic. We have now studied 15 subjects and our preliminary findings are: 1) Epinephrine in the local anesthetic increases plasma epinephrine levels five- to six-fold, without obvious associated increases in heart rate or systolic pressure, 2) Valium sedation attenuates the norepinephrine response to the surgery, again without obvious associated attenuation of heart rate or blood pressure responses, 3) Norepinephrine and epinephrine both increase with dental surgery, as do heart rate, systolic pressure and cardiac output, and 4) Lipids and lipoproteins do not change with dental surgery. These data clearly show that blood pressure does increase with acute stress apparently due to an increase in sympathetic nervous system activity. We will next compare these results with the effects of stress in patients with essential hypertension.

The baroreflex is the most powerful, rapidly acting circulatory homeostatic reflex. There has been debate about whether an abnormality in the reflex characterizes patients with essential hypertension. The discrepancies in previous studies appear to have been related to the different techniques used to measure baroreflex sensitivity. We have measured baroreflex sensitivity via three different techniques, i.e.: 1) Blood pressure and heart rate responses during and after release of the Valsalva maneuver, 2) Blood pressure and heart rate responses to sudden changes in external neck pressure, and 3) Heart rate responses to intravenous injections of vasoconstrictors and vasodilators. We have completed studies in only five patients and our preliminary data suggest the following: 1) Baroreflex sensitivity varies inversely with blood pressure, 2) Baroreflex sensitivity is decreased in both essential and secondary hypertension, and 3) The various techniques for measuring baroreflex sensitivity agree with one another. These findings would suggest that the baroreflex sensitivity changes occurring in patients with essential hypertension are the result of, and not a cause of, their hypertension.

We have developed a sensitive and specific assay for norepinephrine and epinephrine in human plasma. The assay uses a separation with alumina and then high pressure liquid chromatography with electrochemical detection. The method is sensitive to 10 picograms of either catecholamine and yields results which are in close agreement with those of standard radioenzymatic methods. The new method is much faster and less expensive than previous methods. We are currently making use of this method in our studies of the role of the sympathetic nervous system and catecholamines in hypertension. We have also developed a technique for measuring local cerebral glucose metabolism using 2-deoxyglucose in response to electrical stimulation of the carotid sinus nerve in conscious cats. This would allow us to map the central neural pathways mediating the baroreflex. However we are temporarily at a standstill due to apparent injury to the nerve at the time of surgery. We are currently attempting a number of remedies to this problem.

The kallikrein-kinin system is a vasodilator system that we have shown to be reduced in patients with essential hypertension. We have also shown that urinary kallikrein increases during salt deprivation and administration of fludrocortisone. We studied parotid salivary kallikrein to determine whether other types of kallikrein respond to these same maneuvers. It did and it tended to parallel urinary kallikrein in the same subjects. Urinary kallikrein occurs in both bound (prokallikrein) and free forms and approximately equivalent amounts of each form were found in most subjects. Both free and bound kallikrein increased significantly during salt restriction and during fludrocortisone administration. However, we found evidence of decreased conversion of prokallikrein to free kallikrein in subjects with very low levels of free kallikrein and in one patient with hypothyroidism. These findings suggest an enzymatic conversion to activate prokallikrein.

The role of kallikrein in the kidney is still in doubt. Therefore we have undertaken an extensive study of the effect of acute alterations of extracellular volume on urinary kallikrein excretion in conscious rats. The

ECV of female Sprague-Dawley rats was expanded 20% with either normal saline or 5% dextrose in water over a period of one hour at which time the infusion was either stopped or continued to match urinary output. In other studies total body sodium was increased by 15% without concomitant volume expansion with the use of 3% saline; and hyperoncotic albumin infusions were performed in rats during a saline diuresis. Preliminary results show that acute expansion provided the expected significant increase in urinary volume and that sodium loading significantly increased sodium excretion. However urinary kallikrein changed with both significant and insignificant changes in urinary volume, sodium and potassium. Further detailed study of the data are underway to yield final conclusions about the role of kallikrein in the kidney.

We have begun to evaluate an aromatic Tris-amidine which is a potent inhibitor of both glandular and plasma kallikrein. Such an inhibitor would be an important new tool in elucidating the role of the kallikrein-kinin system in both normal physiology and the pathology of hypertension.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZL01-HL-01809-06 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Primary Aldosteronism

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John R. Gill, Jr., M.D. Senior Investigator HE NHLBI

COOPERATING UNITS (if any)

Dr. Maria I. New, Division of Pediatric Endocrinology, Department of Pediatrics, The New York Hospital-Cornell Medical Center, New York, NY

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	2.0	2.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Hyporeninemia, aldosteronism and hypertension, the characteristic features of primary aldosteronism, also characterize the rare disorder, dexamethasone-suppressible aldosteronism. This disorder is familial with an autosomal dominant mode of inheritance which is not HLA linked. As treatment with dexamethasone corrects the aldosteronism and hypertension and restores the responsiveness of the renin-angiotensin system, and as treatment with adrenocorticotrophin (ACTH) produces a sustained increase in aldosterone production and blood pressure, this suggests that the aldosteronism and hypertension are ACTH-dependent. The cause of the ACTH dependence of aldosterone production is unknown but may represent stimulation rather than the usual inhibition by ACTH of 11-hydroxylation and 18-hydroxylation in zona glomerulosa cells. As aminoglutethimide, which blocks selectively steroidogenesis by the zona glomerulosa, pregnancy, which results in an increased production of an aldosterone antagonist, progesterone, and spironolactone, an aldosterone antagonist, correct the hypertension, this suggests that aldosterone alone or in combination with related steroids from the zona glomerulosa mediate the hypertension.

PHS-6040
(Rev. 10-76)

Project Description and Objective:

Patients with dexamethasone-suppressible aldosteronism are characterized by hyporeninemia, hypokalemia, aldosteronism and hypertension. When these patients are treated with dexamethasone, urinary aldosterone decreases to 2 μg/day or less in one to two days followed by correction of the hypokalemia, hyporeninemia, and hypertension. This sequence of events suggests that the overproduction of aldosterone is ACTH-dependent and that the hypertension is aldosterone-dependent.

The response of normal subjects and patients with primary aldosteronism to prolonged treatment with ACTH is an increase in aldosterone production on the first day of treatment followed by a decrease to control values. This fall-off in aldosterone production has been attributed to a decreased conversion of deoxycorticosterone to aldosterone as a result of an ACTH-induced decrease in 11-hydroxylation and 18-hydroxylation in zona glomerulosa cells. This normal pattern of response of aldosterone to ACTH should not occur in patients with dexamethasone-suppressible aldosteronism if aldosterone production is ACTH-dependent. The present studies were performed to test this proposition.

To determine the role of aldosterone in the hypertension of dexamethasone-suppressible aldosteronism, a patient with this disorder was treated with spironolactone and with aminoglutethimide. The latter agent was given during the administration of ACTH which prevented a decrease in cortisol but not in aldosterone.

Methods Employed:

A mother and her two-year-old daughter, both of whom have dexamethasone-suppressible aldosteronism, were given diets which contained 109 and 35 mEq/d of sodium, respectively. Urine was collected daily for the measurements of aldosterone and cortisol. After control observations were obtained, the responses of the mother to ACTH, spironolactone and aminoglutethimide and the response of the daughter to ACTH were determined.

Major Findings:

The effects of pregnancy and of treatment with dexamethasone and spironolactone are shown in the table below:

Treatment or Condition	Blood Pressure mmHg	Serum K^+ mEq/L	Plasma Progesterone ng/ml	Plasma Renin Activity ng/ml/hr	Plasma Aldosterone ng/dl
Untreated	146/97	3.1	0.4	0.8	18
Dexamethasone	120/80	3.5		3.35	<2.0
Spironolactone	114/81	3.7		5.3	22
Pregnancy	112/82	3.8	84	4.0	28

Whereas the improvement with dexamethasone was produced by suppression of aldosterone, the improvement during spironolactone and during pregnancy was produced by antagonism of aldosterone. During the administration of ACTH, treatment with aminoglutethimide decreased aldosterone to very low values (range, 0.2 to 1.6 μg/day) but did not affect plasma cortisol or urinary 17-hydroxycorticosteroids. The decrease in aldosterone was associated with a rise in serum K^+ from 3.3 to 4.1 mEq/L and a fall in blood pressure from 130/90 to 102/77 mmHg. When aminoglutethimide was stopped, aldosterone, serum potassium, and blood pressure returned to pretreatment values. The responses to spironolactone, to pregnancy and to aminoglutethimide demonstrate the important role of sodium-retaining steroids from the zona glomerulosa, such as aldosterone, as mediators of the hypertension.

Treatment of mother and daughter with ACTH produced an increase in urinary aldosterone to maximal values of 30 μg/day (mother) and 12 μg/day (daughter) which were sustained throughout the seven days of treatment and were associated with a further increase in blood pressure. Thus, the observation that ACTH can further stimulate the overproduction of aldosterone that has been shown to be readily suppressible by dexamethasone indicates that ACTH is responsible for this overproduction. This sustained overproduction of aldosterone implies that conversion of deoxycorticosterone to aldosterone is also sustained and that the usual ACTH-induced decrease in 11-hydroxylation and 18-hydroxylation in zona glomerulosa cells does not occur. The mechanism by which ACTH normally inhibits these mitochondrial enzymes of zona glomerulosa cells and the reason that such an inhibition does not occur in dexamethasone-suppressible aldosteronism remain to be determined.

Publications:

1. Dunnick, N.R., Doppman, J.L., Mills, S.R., and Gill, J.R., Jr.: Preoperative diagnosis and localization of aldosteronomas by measurement of corticosteroids in adrenal venous blood. Radiology 133: 331-333, 1979.

2. Auda, S.P., Brennan, M.F., and Gill, J.R., Jr.: Evolution of the surgical management of primary aldosteronism. Ann. Surg. 191: 1-7, 1980.

3. New, M.I., Oberfield, S.E., Levine, L.S., Dupont, B., Pollack, M., Gill, J.R., Jr., and Bartter, F.C.: Autosomal dominant transmission and absence of HLA Linkage in dexamethasone suppressible hyperaldosteronism. Lancet, 550-551, March 8, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-01822-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Catecholamine Metabolism in Bartter's Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	John R. Gill, Jr., M.D.	Senior Investigator	HE NHLBI
OTHER:	C. Raymond Lake, M.D.	Senior Investigator	LCS NIMH

COOPERATING UNITS (if any)

Dept. of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Steroid and Mineral Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	2.0	0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Patients with Bartter's syndrome are resistant to the pressor effects of angiotensin II and norepinephrine. This increase in resistance to angiotensin II and norepinephrine is associated with an overproduction of prostaglandins and is corrected by treatment with a prostaglandin synthetase inhibitor. Recently, we have shown that the increase in pressor resistance to norepinephrine of patients with Bartter's syndrome is associated with an increased excretion of norepinephrine in the urine. This increased excretion of norepinephrine may reflect an increase in activity of the sympathetic nervous system occurring in response to the increase in pressor resistance to norepinephrine. A normal to borderline high excretion of the metabolites VMA and metanephrine and restoration of urinary norepinephrine to normal by inhibition of prostaglandin synthesis supports the hypothesis of an increase in sympathetic nervous system activity mediated by prostaglandins.

Project Description and Objective:

Increased urinary excretions of epinephrine and norepinephrine have been observed in patients with Bartter's syndrome who are resistant to the pressor effects of norepinephrine. If this increased excretion of catecholamines indicates an increase in sympathetic activity and does not reflect a decrease in metabolism of norepinephrine then catecholamine metabolites should be normal or increased. Furthermore, if the increase in sympathetic activity occurs in response to an increase in synthesis of vasodilator prostaglandins by blood vessels, then treatment with a prostaglandin synthetase inhibitor which corrects the pressor responsiveness should lower urinary norepinephrine toward normal.

Methods Employed:

Seven patients with Bartter's syndrome were given a diet which contained 109 mEq per day of sodium. Urine was collected daily for the determination of norepinephrine, epinephrine, normetanephrine, metanephrine and vanillylmandelic acid. After four days of control observations, indomethacin, 150 mg/day, was given for four days. The control values are compared to values for seven normal age-matched subjects.

Major Findings:

The excretion of epinephrine, norepinephrine, metanephrine and 3-methoxy-4-hydroxymandelic acid (VMA) by three patients with Bartter's syndrome and the effects of treatment with indomethacin are presented as means ± S.E. in the table below.

	CONTROL			
Patient	Epinephrine (μg/day)	Norepinephrine (μg/day)	Metanephrine (mg/day)	VMA (mg/day)
1	19	386	0.91	1.45
2	35	273	1.08	2.68
3	14	100	1.00	2.60
Mean ± SEM	23±7	253±95	1.00±0.06	2.24±0.41

INDOMETHACIN

Patient	Epinephrine (μg/day)	Norepinephrine (μg/day)	Metanephrine (mg/day)	VMA (mg/day)
1	6	219	0.29	0.83
2	42	194	0.94	1.77
3	14	20	0.79	2.70
Mean ± SEM	21±12	144±66	0.67±2.2	1.77±0.62

Mean urinary norepinephrine was 253 ± 95 μg/day (normal, 73.3 ± 11.2 μg/d), and mean urinary epinephrine was 22.9 ± 7.0 μg/day (normal, 27.8 ± 3.4 μg/d), mean metanephrine was 1.00 ± 0.06 mg/day (normal, <1.0 mg/d) and mean VMA 2.24 ± 0.41 mg/day (normal, 0.7 - 6.8 mg/d). Treatment with indomethacin decreased epinephrine by 8%, norepinephrine by 43%, metanephrine by 33% and VMA by 20%. These results indicate that the increased rate of excretion of norepinephrine is associated with a normal rate of excretion of metabolites. Thus, the supranormal values for norepinephrine cannot be explained by subnormal metabolism and are best explained by an absolute increase in catecholamine excretion, presumably the result of an increase in sympathetic activity. The decrease in norepinephrine and metabolites during treatment with indomethacin suggests that the increase in sympathetic activity was a response to a prostaglandin-dependent increase in vascular resistance to norepinephrine.

Publications:

1. Güllner, H.G., Bartter, F.C., Cerlitti, C., Smith, J.B., and Gill, J.R., Jr.: Prostacyclin overproduction in Bartter's syndrome. The Lancet, October 13, 1979, 767-769, 1979.

2. Brouhard, B.H., Gill, J.R., Jr., Yun, J.C.H., Kelly, G.D., and Bartter, F.C.: Prostaglandin dependent and independent effects of bradykinin on renal function in the dog. Renal Physiology, Basel 2: 44-53, 1979-80.

3. Gill, J.R., Jr.: Bartter's syndrome: Ann. Rev. Med. 31: 405-419, 1980.

4. Düsing, R., Gill, J.R., Jr., Bartter, F.C., and Güllner, H.G.: The effect of potassium depletion on urinary prostaglandins in normal man. In: Advances in Prostaglandin and Thromboxane Research, (eds.) B. Samuelsson, P.W. Ramwell and R. Paoletti, Raven Press, New York, 1980, pp. 1189-1192.

5. Güllner, H.G., Gill, J.R., Jr., Bartter, F.C., Lake, C.R., and Lakatua, D.J.: Correction of increased sympathoadrenal activity in Bartter's syndrome by inhibition of prostaglandin synthesis. J. Clin. Endo. Metab. 50: 857-861, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01924-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Separation of Renin-Like Activity from Non-specific Acid Proteases in Vessel Wall

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	M. Fordis, M.D.	Staff Associate	NHLBI	HE
OTHER:	J. Megorden	Biological Aide	NHLBI	HE
	T. Ropchak, B.S.	Biol. Lab. Tech.	NHLBI	HE
	H.R. Keiser, M.D.	Deputy Chief	NHLBI	HE

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION

TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of renin-like activity (RLA) in vessel wall have been plagued by low levels of RLA and nonspecific acid proteases (NAP). Protease inhibitors and affinity chromatography were used to address these problems. The new methods permitted complete separation of RLA from NAP. When sodium depleted rats were studied before nephrectomy and at 2 hours and 24 hours post-nephrectomy, the vascular RLA (devoid of proteolytic activity) fell in parallel with the plasma renin activity (PRA). In animals studied 24 hr after bilateral nephrectomy and 15 hours after hemorrhage the vascular RLA was increased over controls which were nephrectomized and not hemorrhaged. In conclusion the method permitted complete separation of RLA from NAP. After nephrectomy alone, the parallel fall in RLA and PRA suggests that RLA may arise from the plasma. However, the marked increase in RLA after hemorrhage indicates that vascular RLA can be independent of PRA.

PHS-6040
(Rev. 10-76)

Objective: The present studies investigate the role of the renin-angiotensin system (RAS) in the local control (as opposed to hormonal) of vascular tone. The studies to date have focused on 1) the development of methods to isolate small resistance vessels as well as large vessels for study 2) the establishment of assays for renin concentration, and proteolytic activity 3) the development of methods to separate nonspecific acid protease activity (NAP) from renin-like activity (RLA) and 4) physiologic evidence for local production of RLA distinct from plasma renin activity (PRA) and from NAP.

Materials and Methods:

1. Isolation of large vessels: While under ketamine anesthesia animals were exsanguinated and then perfused with cold saline. Standard surgical techniques were used to isolate and remove the aorta from the ascending aorta to the bifurcation of the femorals. Loose connective tissue was removed and the aortas frozen.

2. Isolation of small resistance vessels (microvessels): Microvessels from rat brain were isolated by one of two techniques. In one technique brains were homogenized in physiologic buffer and filtered through various meshes of nylon screen. Successive homogenizations with homogenizers of specified clearances to be followed by filtrations through specified nylon mesh produced fractions enriched in muscular vessels and capillaries. Yields were small (milligrams) and some white and grey matter contaminated the final product. In another technique rat brain microvessels (MV) were isolated by ultracentrifugation of the homogenate on sucrose gradients. Yields were increased and white matter contamination removed. Assays for gamma-glutamyl transpeptidase and angiotensin converting enzyme are planned to permit quantification of MV purity. As in the isolation of large vessels, MV were isolated only after the rat had been exsanguinated and perfused with cold saline.

3. Renin Assay: Initially the renin assay available from Squibb was tested. The method involves incubation at neutral pH in the presence of metal chelators, EDTA, 8-hydroxyquinoline sulfate, and dimercaprol. Antibody, standard, and charcoal were supplied by Squibb. In the separation of bound Angiotensin I from free Angiotensin I significant misclassification error occurred as the charcoal could not be compacted despite centrifugation at high g-forces. Assay parameters for curves with bound/total ratios (B/T) of 50% were--

	Nonspecific	Slope	ED$_{50}$	Minimal Detectable Dose (MDC)	Coefficient of variation of curve of MDC (CV-MDC)	Optimal Coeff. of variation near ED$_{50}$ (CV-ED$_{50}$)
$\bar{X}$ ± 1 SD	520±43	-.844±.104	275±118	50-100pg/ml	38±9	24±6

These parameters were clearly unacceptable. High nonspecific counts reflected misclassification error. Slopes were variable, MDC and ED_{50} were too high. The coefficient of variation which described the optimal portion of the curve was too large. The following modifications were made.

Antibody was obtained from Dr. F. Katz. Charcoal was prepared with dextrose. Angiotensin I standard was purchased from Squibb and labeled angiotensin I was purchased from New England Nuclear. Assays with B/T ratios of 50% are described by the following parameters:

	Nonspecific	Slope	Ed_{50}	MDC	CV-MDC	$CV\text{-}ED_{50}$
$\bar{X}$±1SD	133±39	-1.09±.054	137±14	12.5-25pg/ml	29±9	7±2

Quality controls were prepared from plasma obtained from a human volunteer. With the modified assay, quality controls run 10.46 ± 1.09 ($\bar{X}$ ± 1SD) ng AI/ml/3hr. Experiments were performed to examine the ability of various EDTA inhibitors to protect AI generated during incubation. For long tissue renin incubations EDTA, neomycin and phenylmethylsulfonyl fluoride were superior. Better than 80% of the angiotensin I generated was recovered after 24 hours of incubation.

Plasma from rats 36-48 hours after nephrectomy was used as a source of renin substrate. All substrate or plasma samples were collected and centrifuged at room temperature to remove the cellular components. Plasma was handled at room temperature until acidification to pH 5.9 for the assay to minimize possible cryoactivation.

4. Proteolytic Assays: The conditions which maximize AI production by renin are conditions suitable for proteolysis. Nonspecific acid proteases (NAP) such as the cathepsins can produce angiotensin I immunoreactive material from renin substrate. Such proteolysis can obscure the low levels of renin-like activity which might be present in tissues such as vessel wall. Assays to detect proteolytic activity were developed. Homogenates were incubated with bovine Hgb at pH 3.5 and the non-TCA precipitable cleavage fragments measured with the Bradford Coomassie Blue protein assay. This method permitted detection of 10^{-1} to 10^{-2} units of cathepsin D. Under the incubation conditions for the renin assay, 10^{-2} units of cathepsin can produce measurable immunoreactive AI. Sensitivity could not be increased by G-25 sephadex filtration of the substrate. The recent availability of hemoglobin substrate tagged with trituim by Dr. French Anderson permitted detection of 10^{-3} units of cathepsin D. The increased sensitivity of the assay allows the detection of any quantitatively significant proteolytic activity in samples with measurable renin-like activity. Proteolytic activity at various pH's and times were measured to determine the assay incubation time and pH.

5. Separation of Renin-like Activity from Nonspecific Acid Protease Activity. Columns of bovine hemoglobin coupled to Sepharose-4B were prepared and experiments were performed to determine the conditions necessary to separate renin-like activity from nonspecific acid protease activity. Under the appropriate conditions renin-like activity in tissue could be completely separated from proteases bound to hemoglobin.

Results:

Experiments were performed to investigate the renin-like activity in large vessels and in small resistance vessels. In the discussion below all AI generating activity is referred to as RLA whether it represents renin, a renin-like enzyme, or NAP.

1. Large Vessels: The aortas of rats were collected as described above and pooled. Both aortic RLA and PRA were measured in Na-depleted rats before nephrectomy and at 2 hours and 24 hours post-nephrectomy (PN). Chromatography demonstrated two peaks of RLA--peak 1 contained no proteolytic activity while peak 2 did. The proteolytic activity represents NAP not irreversibly inhibited above. Prior to nephrectomy, RLA was 34.3 ng/AI/hr in peak 1 and 0.40 ng AI/hr in peak 2. At 2 hours PN, RLA in peak 1 had fallen to 4.4 ng AI/hr and at 24 hours peak 1 was no longer detectable. PRA fell from 31.4±2.64 ($\bar{X}$±SEM) ng/ml/hr before nephrectomy to 2.89±1.51 ng/ml/hr at 2 hrs PN and to 0.23 ±0.08 ng/ml/hr at 24 hours PN. To distinguish peak 1, RLA from PRA, animals were hemorrhaged (15% of TBV) nine hours after bilateral nephrectomy. In preliminary experiments, 24 hours PN, peak 1 RLA from hemorrhaged animals was 2.7 ng/AI/hr while PRA averaged .18±.02 ng AI/ml/hr. In conclusion, this method allows the separation of RLA (peak 1) from NAP (peak 2). After nephrectomy alone, the parallel fall of RLA (peak 1) with PRA suggests that RLA (peak 1) arises from the plasma. However, the marked increase in RLA (peak 1) after hemorrhage indicates that vascular RLA can be independent of PRA.

2. Microvessels: Several experiments were performed with pooled fractions of microvessels. No activity has been detected. Because of the small quantities of protein in microvessels fractions, activity may disappear with dilution during chromatographic steps. The methods are now being scaled down to reduce this problem. Additionally RLA may be degraded by proteases in the initial steps of microvessels isolation. The procedure will be modified to inhibit proteases as soon as possible in the microvessel isolation.

Conclusion and Plans:

Methods have been developed to separate NAP from renin-like enzymes. The activity in large vessels may be physiologically important in control of vascular tone. In the model described above aortic RLA without proteolytic activity increased independent of PRA. The model will permit 1) the characterization of RLA in vascular wall apart from that in the plasma. 2) Study of regulation including evaluation of the role of the sympathetic nervous systems 3) evaluation of the anatomy of the system with immunofluorescence (i.e. the location of substrate, renin-like enzyme, and converting enzyme).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01925-02

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Study of Aromatic Tris-Amidine as an Inhibitor of Kallikrein

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Eric Marks, M.D.	Staff Associate	NHLBI HE
OTHER:	Harry Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	NHLBI HE
	Marian E. Warner, BS	Biologist	NHLBI HE

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.2	0.1	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES XX☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

I-RT-36, an aromatic tris-amidine compound, has been demonstrated to inhibit porcine pancreatic and human plasma kallikrein, with a K_i in the 10^{-8} range. The purpose of the present study was to evaluate this inhibitory capacity both in vivo and in vitro using the rat as an experimental model. Initial studies demonstrated blockade of the expected hypotensive response provoked by the injection of kallikrein isolated from rat urine, when the experimental animal was pre-treated with the I-RT compound. In work with guinea pig ileum, addition of the tris-amidine to the bath containing a kininogen source and kallikrein blocked the expected kinin induced smooth muscle contraction.

Objective: The purpose of this study was to evaluate the tris-amidine compounds as inhibitors of kallikrein. As presently available inhibitors of kallikrein are not specific, there is great need for a selective inhibitor in the study of the kallikrein-kinin system. The tris-amidine compounds have demonstrated K_i in the 10^{-8} range, and are more active against kallikrein, than trypsin or thrombin. It was necessary to prove that the compounds were active against the system in the rat, as this is our model for additional kallikrein studies. The compound has a M.W. of 868.71 which should effect its immunogenicity. A selective inhibitor would allow more accurate studies of a system whose interactions are far reaching.

Methods:

1. Preparation of kallikrein: Urine was collected from salt depleted rats, housed in metabolic balance cages. The urine was collected under toluene, filtered, spun, and quick frozen. The collected urine was concentrated with an Amicon filter, diluted with column buffer and dialyzed for 24 hours. This material was run over a DE-52 ion-exchange column, and subsequently a Trasylol affinity column. This provided separation from esterase A activity. The resultant material had no intrinsic activity in the bio-assay preparation.

2. Preparation of pseudoglobulin fraction: Rats were exsanguinated; blood was collected in heparinized containers and spun to obtain plasma. The plasma was treated with ammonium sulfate. The pseudoglobin fraction served as a kininogen source.

3. Guinea pig ileum was used for the bioassay.

4. Male SD rats (25-300 grams) were surgically prepared for femoral vessel cannulation, infusion of micro-liter quantities of materials was performed with a modified Hamilton syringe. BP was recorded with a Statham P23Db transducer and a Sanbourn 150.

Results:

1. Evaluation of Pseudoglobulin as kininogen source. Bioassay in rat uterus revealed slight inhibition of kinin generation with the kallikrein dose of 0.1μ gms. The pseudoglobulin preparation had no kininase activity, but it appears to contain either a kallikrein inhibitor or an inactivating enzyme. The pseudoglobulin was heated to 56°C for 1 hour in an attempt to inactivate any inhibitor and the material is to be re-tested.

2. In vitro-testing: The Guinea pig ileum bioassay was used to evaluate potency and pattern of inhibition. Rat urinary kallikrein (1 μgm) added to the bath containing 100 μl of pseudoglobulin, produced an average response of 26 mm. deflection. When 5 μgm of IRT-36 was added to the pseudoglobulin-kallikrein solution, the intensity of the response fell to 8.5 mm (average 67% reduction) and the onset of action was delayed by a factor of five. This pattern suggests that the IRT acts as a competitive inhibitor. IRT, pseudoglobulin, and rat urinary kallikrein had no effects by themselves.

3. In vivo testing: Rat urinary kallikrein (1 μgm) lowered blood pressure when injected IV into anesthetized rats, as did 0.7 ml of fresh rat urine. Pre-treatment with a dose of 0.1 mg of IRT-36 did not inhibit this vasodepressor response. The effect of the inhibitor was evaluated in the two-kidney Goldblatt model of renovascular hypertension. IRT at a dose of 0.1 mg I.V. depressed blood pressure. This effect was transient and pressure returned to pre-treatment levels by one hour. Rechallange with IRT again produced a vasodepressor effect. This fall in blood pressure was noted in normal rats, but to a lesser extent. The vasodepressor effect of esterase A was not blocked by pre-treatment with IRT.

Significance of Findings:

The in vitro effects of IRT-36 suggest that it is a potent inhibitor of kallikrein. When IRT was pre-incubated with pseudoglobulin, this inhibitory effect was lost, similar to the course of events in the whole animal. This may be an effect of non-specific protein binding which prevents the inhibitor from making contact with kallikrein. It is possible that a significant increase in dose level would overcome this effect. The direct action of IRT on blood pressure was unexpected, and no evidence of intrinsic activity appears in the in vitro testing. However smooth muscle relaxation would not be readily apparent in the ileum preparation.

The need for a specific inhibitor of kallikrein is obvious. A specific inhibitor would allow a much cleaner approach in defining the role of the kallikrein-kinin system.

Proposed course of the study:

1. To evaluate the direct action of the IRT in vivo, through the use of the isolated rat hind limb preparation.

2. Evaluate dose-response relationship in vivo.

3. Complete in vitro bioassays to determine relative potency of the inhibitor.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01926-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Studies of salivary and urinary kallikrein in humans

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	David Horwitz, M.D.	Senior Investigator	NHLBI HE
OTHER:	Harry Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	NHLBI HE
	David Proud, Ph.D.	Visiting Fellow	NHLBI CH
	John Pisano, Ph.D.	Head, Sec. on Physiological Chemistry	NHLBI CH

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Salivary and urinary kallikrein were measured in subjects receiving different levels of dietary salt and fludrocortisone. Urinary kallikrein occurred as prokallikrein and free kallikrein. Salivary as well as both forms of urinary kallikrein increased with salt deprivation and fludrocortisone.

Objectives: We have been interested in kallikreins because they liberate vasodilator peptides and because hypertensive patients have been found to have reduced urinary excretion of free kallikrein. We have shown that urinary kallikrein increases during salt deprivation and administration of fludrocortisone; in an effort to determine whether other types of glandular kallikrein respond to these maneuvers, we measured parotid salivary kallikrein during such stimulation. Parotid saliva was studied because salivary glands are rich in kallikrein, because salivary function is modified by various kinins and because quantitative collection is feasible.

Urine contains both free and prokallikrein. Studies were initiated to determine the extent to which low levels of urinary free kallikrein may reflect deficiencies of prokallikrein or its conversion to the free form.

Methods: Urinary assays for normal subjects were performed on 24 hour urine samples obtained on the last two days of one week periods of uniform intake of dietary salt. Equilibration was demonstrated by the finding that urinary excretion approximated dietary intake.

The radiochemical esterolytic assay of Beavan et al. was used to measure urinary kallikrein; assays were performed both in untreated gel-filtered samples and after trypsin activation to convert inactive prokallikrein to active or free kallikrein; the difference between kallikrein levels in unactivated and activated samples was designated as prokallikrein whereas kallikrein in unactivated samples was called free kallikrein.

Salivary kallikrein was assayed by radio-immunoassay because of the possibility that salivary esterases might interfere with the esterolytic assay of Beavan et al.

Parotid saliva was collected before supper using a Lashley cup during standard stimulation with small sips of lemon juice once a minute. Concentrations and flow were determined from timed samples collected over ten minutes.

Statistical analyses were by Student's t-test and by analysis of variance.

Results: Urinary kallikrein: Eighteen unselected normal volunteers were studied at the end of one week periods during which they received 109 meq,9 meq or 259 meq of dietary salt daily. The sequence of diets was varied. Urinary kallikrein occurred in both bound (prokallikrein) and free forms; approximately equivalent amounts of each form were found in most subjects. Both free and bound kallikrein increased significantly during salt restriction and during administration of the salt-retaining steroid,fludrocortisone.

Two hypertensive and two normotensive subjects with no measurable levels of free urinary kallikrein on intermediate levels of dietary salt were selected for study. Three showed normal excretion of prokallikrein while the fourth showed minimal prokallikrein; each showed increased excretion of prokallikrein during salt restriction.

EFFECTS OF DIFFERENT LEVELS OF DIETARY SALT AND ADMINISTRATION OF FLUDROCORTISONE ON MEAN DAILY EXCRETION OF URINARY KALLIKREIN

Assay	Diet (meq Na/day)							
	9	109	259	N		Control	Fludro-cortisone	N
Free Kallikrein (T.U./day)	16.6	8.7*	5.1*	18		7.3	16.9*	13
Prokallikrein (T.U./day)	8.0	6.1*	5.2*	18		5.3	10.3*	13

* p<.05 for comparison with a 9 meq diet or control

Salivary Kallikrein: The average concentrations of parotid kallikrein and potassium of 16 normal volunteers increased significantly during salt restriction whereas that of sodium decreased. Changes in output were in the same direction but because of the variability of measurements of flow, statistical significance was not reached; there were no significant differences in average flow in the different periods.

EFFECTS OF CHANGES IN DIETARY SALT ON PAROTID SALIVA OF SIXTEEN NORMAL VOLUNTEERS

		Salt	Dietary
	9 meq/d	109 meq/d	259 meq/d
Concentration of Kallikrein (RIA) μg/ml	2.25	1.65$^+$	1.85*
Concentration of Potassium meq/L	25.7	22.7$^+$	21.2$^+$
Concentration of Sodium meq/L	19.6	24.5*	26.1$^+$
Output of parotid kallikrein μg/min	1.43	1.17	1.31
Flow cc/min	0.66	0.74	0.85

$^+$ p<.01 in comparison with 9 meq period
* p<.05 in comparison with 9 meq period

Administration of fludrocortisone 0.6 mg/day for one week to ten normal volunteers receiving 109 meq of salt daily caused a significant rise in the concentration of parotid kallikrein but potassium, sodium and flow were not significantly altered.

EFFECT OF FLUDROCORTISONE ON PAROTID SALIVA OF TEN NORMAL VOLUNTEERS

	109 meq Na/d	109 meq Na/d + Fludrocortisone
Concentration of Kallikrein (RIA) µg/ml	1.84	2.43*
Concentration of Potassium meq/L	23.4	23.5
Concentration of Sodium meq/L	18.8	17.2
Output of kallikrein (RIA) µg/min	1.10	1.52
Flow cc/min	.71	.76

* p<.01

Endocrine Effects

Following removal of an aldosterone-producing adenoma, concentration and output of parotid kallikrein of one patient fell strikingly from supra-normal levels; parotid potassium and sodium fell and rose respectively; urinary excretion of aldosterone fell from elevated levels and plasma renin activity rose from suppressed values.

CHANGES IN AVERAGE VALUES FOR PAROTID SALIVA AFTER REMOVAL OF AN ALDOSTERONE-PRODUCING ADENOMA (ONE PATIENT)

Period	Kallikrein µg/ml	Sodium meq/L	Potassium meq/L	Flow cc/min
pre surgery	6.1	17	32	.74
post surgery	1.0	41	23	.72

One patient was studied when replacement therapy with 75 mcg/day of triiodothyronine was suspended to permit a search for possible metastases of a thyroid malignancy. While hypothyroid she showed a pronounced fall in the ratio of free to bound urinary kallikrein and a fall in the concentration and output of parotid kallikrein; urinary and plasma aldosterone showed little change.

EFFECTS OF HYPOTHYROIDISM ON URINARY AND PAROTID KALLIKREIN (ONE PATIENT)

Treatment	Plasma T3	Urinary Kallikrein T.U./day			Parotid			
		Free	Bound	Aldosterone µg/day	Kallikrein µg/ml	Sodium meq/L	Potassium meq/L	Flow cc/min
Triiodothyronine	233	10.2	9.0	6.2	1.7	28.0	29.1	1.08
0	36	4.2*	12.8	5.2	1.2*	33.3*	26.6*	1.07

* p<.025 for comparison with period of thyroid replacement

Significance of findings: Urinary kallikrein exists in an inactive as well as an active form. Both forms are increased during salt deprivation and administration of fludrocortisone. Evidence for decreased conversion of inactive to active kallikrein was found in subjects with spontaneously low levels of kallikrein and in hypothyroidism.

Stimulation of kallikrein levels by salt-retaining steroid was shown to occur with salivary as well as urinary kallikrein suggesting that this may be a general response of glandular kallikrein.

Proposed course of project: Additional subjects with hypothyroidism and aldosterone-producing adenomas will be studied.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01928-01

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Effects of carotid sinus nerve stimulation on local cerebral glucose metabolism in conscious cats.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	David S. Goldstein, M.D., Ph.D.	Clinical Associate	NHLBI	HE
OTHER:	Joseph DiMicco, Ph.D.	Staff Fellow	NIMH	CS
	Joseph E. Pierce, D.V.M.	Section Chief	NHLBI	LAMS
	Louis Sokoloff, M.D.	Laboratory Chief	NIMH	CM
	Harry R. Keiser, M.D.	Deputy Chief	NHLBI	HE

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: .30	PROFESSIONAL: .30	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have developed a technique for measuring local cerebral glucose metabolism using 2-deoxyglucose in response to electrical stimulation of the carotid sinus nerve in conscious cats. The technique appears to be limited by damage to the nerve at the time of surgery.

Objectives: The baroreflex is the most potent, rapidly-acting circulatory homeostatic mechanism for controlling blood pressure. Stretch receptors in the carotid sinus area respond to increases in blood pressure by increased afferent activity along the carotid sinus nerve, resulting in reflex decreases in heart rate and peripheral resistance which tend to bring blood pressure toward normal.

The central neural pathways mediating the baroreflex are poorly described. The 2-deoxyglucose (2DG) technique offers the potential for mapping out these areas by identifying brain centers which are metabolically active. We plan to stimulate the carotid sinus nerve and measure local glucose uptake - and therefore metabolic activity - using the 2DG technique. Since anesthesia grossly distorts the baroreflex, the experiment requires conscious subjects.

Methods: Using sterile technique, we implant femoral arterial and venous catheters in anesthetized, artifically respired cats. We tunnel the catheters subcutaneously so that they exit behind the neck. The carotid sinus nerve on one side of the neck is identified and prepared for stimulation. The nerve is surrounded by a specially fabricated bipolar electrode, with the leads tunneled subcutaneously to exit behind the neck.

After recovery from anesthesia (1 to 2 days), the cat while awake is restrained in a sling and the function of the electrode tested by measuring blood pressure and heart rate responses to electrical stimulation of the carotid sinus nerve. When nerve function has been verified, the 2DG technique is carried out with or without concurrent stimulation of the nerve. The C^{14} labelled 2DG is injected intravenously, with serial arterial samples drawn for concentration calculations during the subsequent 40 minutes. The cat is then sacrificed, the brain extracted, and metabolic activity computed using a computerized radioautographic procedure.

Results and their Significance: We have been successful so far with only one cat of approximately 12. In that cat, carotid sinus nerve stimulation did not produce any obvious unilateral increase in 2DG uptake in the nucleus tractus solitarius, the area known to be the location of the first synapse of the baroreflex. In the remaining cats, post-operative death, failure to elicit a hypotensive response to nerve stimulation on the day planned for 2DG administration, or excessive blood glucose levels have prevented completion of the preparation.

Proposed Course of Study: We will be meeting with Mr. Sullivan (of Instrument Fabrications) to determine if revision of the electrode will minimize neural damage. If unpreventable, irreversible trauma accompanies the dissection and electrode implantation, and the project will be abandoned.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01929-01

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Lipoprotein and catecholamine responses to dental surgery.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	David S. Goldstein, M.D., Ph.D.	Clinical Associate	NHLBI	HE
OTHER:	Richard Gregg, M.D.	Clinical Associate	NHLBI	MD
	James Sweet, D.D.S.	Staff Oral Surgeon	NIDR	Clinic
	Raymond Dionne, D.D.S., Ph.D.	Staff Fellow	NIDR	NAB
	Ronald Dubner, D.D.S., Ph.D.	Branch Chief	NIDR	NAB
	Richard Gracely, Ph.D.	Research Psychologist	NIDR	NAB
	H. Bryan Brewer, M.D.	Branch Chief	NHLBI	MD
	Harry R. Keiser, M.D.	Deputy Chief	NHLBI	HE

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.30	.30	

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have measured cardiovascular (heart rate, blood pressure, and cardiac output), biochemical (plasma norepinephrine, epinephrine, growth hormone, cortisol, cholesterol, triglyceride, and lipoprotein), and psychological (pain, anxiety) responses to wisdom tooth extractions in healthy young adults.

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Objectives: Undergoing wisdom tooth extractions is a real-life, acute emotional stress. By measuring plasma norepinephrine levels - reflecting sympathetic nervous system activity and cardiovascular variables - we hope to characterize the response to non-physical stress in normal persons, in order to provide a basis for comparison with patients with hypertension and other cardiovascular diseases, in whom excessive sympathetic responsiveness to stress may have etiological significance. In the current study, we wish to test these hypotheses: (1) The stress of dental surgery systematically alters lipoprotein and catecholamine levels; (2) Catecholamine levels correlate with the amount of self-reported anxiety or experienced pain; (3) Lipoprotein and catecholamine responses to stress are inter-correlated; (4) Catecholamine levels correlate with results of non-invasive cardiovascular physiological measurements; (5) Intravenous diazepam sedation attenuates the cardiovascular and catecholamine responses to dental surgery; and (6) Epinephrine in the local anesthetic increases plasma epinephrine levels, with correlated changes in heart rate and blood pressure.

Methods: Blood drawn through an indwelling intravenous needle has been assayed for the above biochemical parameters in healthy young adults a few days prior to surgery, and during pre-operative, operative, and post-operative conditions, for each of two sets of wisdom tooth extractions. For the first operation, patients were randomly assigned to receive or not to receive intravenous diazepam (Valium) sedation five minutes prior to the local anesthesia; for the second, they were randomly assigned to receive or not to receive epinephrine in the local anesthetic. For the first operation, all patients received epinephrine in the local anesthetic, and for the second, all received diazepam sedation. Blood pressure has been measured using an Arteriosonde intraoperatively, and manually in the baseline and post-operative conditions. Impedance cardiography has been used as a non-invasive index of cardiac output. Experienced pain and anxiety have been measured using standardized questionnaires.

Results and their Significance: Although preliminary, data obtained from 15 patients yield several findings: (1) Epinephrine in the local anesthetic increases plasma epinephrine levels five- to six-fold, without obvious associated increases in heart rate or systolic pressure; (2) Valium sedation attenuates the norepinephrine response to the surgery, again without obvious associaed attenuation of heart rate or pressure responses; (3) Norepinephrine and epinephrine both increase with dental surgery, as do heart rate, systolic pressure, and cardiac output; and (4) lipids and lipoproteins do not change with dental surgery. In order to measure the catecholamine levels, a liquid chromatographic assay technique using electrochemical detection was perfected and validated against the radioenzymatic technique. This research is discussed separately.

Proposed Course of Project: This project will only require 20 patients, as opposed to the anticipated 40. The project should therefore be completed by fall, 1980, and a new project, involving patients with hypertension or other cardiovascular diseases, begun.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01930-01

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparison of techniques for measuring baroreflex sensitivity in hypertension.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	David S. Goldstein, M.D., Ph.D.	Clinical Associate	NHLBI HE
OTHER:	Rodman Turner, B.A.	Biologist	NHLBI HE

COOPERATING UNITS (if any)

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Experimental Therapeutics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.40	.40	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have measured baroreflex sensitivity in patients with essential and secondary hypertension. Early results suggest the following: (1) Baroreflex sensitivity varies inversely with blood pressure; (2) Baroreflex sensitivity is decreased in both essential and secondary hypertension; and (3) The various techniques for measuring baroreflex sensitivity agree with one another.

Objectives: Since the baroreflex is the most powerful, rapidly acting circulatory homeostatic reflex, we have investigated whether an abnormality in this reflex characterizes patients with essential hypertension. We have measured baroreflex sensitivity using three techniques, in order to answer these questions: To what extent are the obtained reflex sensitivities consistent across techniques? Are alterations in baroreflex function specific for essential hypertension? And are the alterations reversible when blood pressure is decreased to normal? Previous studies have disagreed about whether or not patients with essential hypertension show abnormal baroreflex function, and the discrepancies appear to have been related to the technique used to measure baroreflex sensitivity.

Methods: The three techniques used to assess baroreflex sensitivity have been: (1) blood pressure and heart rate relationships during and after release of the Valsalva maneuver; (2) blood pressure and heart rate responses to sudden changes in external neck pressure; and (3) heart rate responses to intravenous injections of vasoconstrictors and vasodilators. Baroreflex sensitivity has been defined as the change in interbeat interval per unit change in systolic pressure, for the first and third techniques; and the change in interbeat interval or in systolic pressure per unit change in neck cuff pressure, for the second technique. The vasoconstrictor used in this study has been phenylephrine, and the vasodilator nitroglycerine. Blood pressure has been measured on a beat-to-beat basis via an indwelling arterial catheter.

BAROREFLEX SENSITIVITY IN PATIENTS WITH HYPERTENSION AND IN NORMOTENSIVE CONTROLS

				Baroreflex sensitivity						
Patient	Diagnosis	Val. strain	Val. release	phenyl.	nitro.	cuff R-R -p	cuff R-R +p	cuff BPs -p	cuff BPs +p	Baseline BPs
S.H.	Healthy	6.33	15.50	11.40	9.24	-8.82	-2.37	0.24	0.32	102
S.B.	Ess. HTN	2.95	4.10	14.60	6.82	-3.12	0.41	0.04	0.16	122
S.S.	Renal HTN	2.62	2.59	-----	1.81	-1.35	-0.91	0.28	0.21	189
D.C.	Healthy	5.15	5.83	14.80	2.33	-2.54	-0.22	0.06	0.03	114
K.P.	Ess. HTN	2.91	3.89	9.42	3.81	-2.08	0.59	0.15	0.02	133

Notes: Ess. HTN = essential hypertension; Renal HTN = renovascular hypertension; Val. strain = change in R-R interval per mm Hg change in systolic pressure during the decrease in pressure associated with the Valsalva maneuver; Val. release = change in R-R interval per mm Hg change in systolic pressure during the increase in pressure associated with release of the Valsalva maneuver; phenyl. = change in R-R interval per mm Hg change in systolic pressure during the pressure increase produced by bolus intravenous injection of phenylephrine; nitro. = change in R-R interval per mm Hg change in systolic pressure during the pressure decrease produced by intravenous injection of nitroglycerine; cuff = change in R-R interval or in systolic pressure produced by increases (+p) or decreases (-p) in external neck pressure. Patient S.S. did not receive phenylephrine due to her high baseline systolic pressure.

Results and their Significance: Action on this project was delayed due to the time required for design and fabrication of the apparatus used to produce sudden changes in external neck pressure; and due to the submission of an amendment to the research protocol allowing bolus injections, rather than slow infusions, of the vasoactive substances. Five patients have undergone the baroreflex testing procedure - 2 normotensive controls, 2 patients with essential hypertension, and 1 patient with renovascular hypertension. The results are summarized in the table. Several trends in the data are consistent with the following hypotheses, although the results are much too preliminary for firm conclusions: (1) Baroreflex sensitivity varies inversely with blood pressure; (2) Baroreflex sensitivity is decreased in both essential and secondary hypertension; and (3) The various techniques for measuring baroreflex sensitivity agree with one another. These findings, if verified in the remainder of the study, would suggest that the baroreflex sensitivity changes occurring in patients with essential hypertension are the result of, and not a cause of, their high blood pressure.

Proposed Course of Project: We will continue this project, using the amended protocol, to a total of about 25 patients.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01931-01

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of Alterations of Extra-Cellular Volume on Urinary Kallikrein Excretion

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	Eric Marks, M.D.	Staff Associate	NHLBI HE
OTHER:	Harry Keiser, M.D.	Deputy Chief, Hypertension-Endocrine Branch	NHLBI HE
	Marian E. Warner, BS	Biologist	NHLBI HE

COOPERATING UNITS (if any)

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Experimental Therapeutics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.9	0.9

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The extra-cellular volume of female Sprague-Dawley rats was expanded by 20% with either NS or D5W over a period of one hour, at this point the infusion was either stopped or continued to match urinary output. In other studies, total body sodium was increased by 15% without concomitant volume expansion, through the use of 3% saline, and hyperoncotic albumin infusions were performed in rats undergoing a saline diuresis. The purpose of these manipulations was to evaluate the role of the kallikrein system in adaptation to acute alterations in extra-cellular volume and salt concentrations. Urine was collected over timed periods and tested for [Na], [K], and kallikrein. A radioimmunoassay was used to measure kallikrein. Initial results reveal changes in kallikrein during expansion. The relationship of kallikrein to the other measured variables is not clear at this time.

Objective: A relationship between urinary excretion of kallikrein and salt handling by the kidney has been postulated for some time. Previous studies have reported conflicting results and model systems have not been standardized. This study was designed to evaluate the response of the kidney in terms of kallikrein excretion, to acute volume expansion, performed in conscious animals, similarly prepared and expanded. Kallikrein has also been tentatively assigned a role in blood pressure regulation, therefore its interaction with volume, salt, and the kidney is of great interest.

Methods:

1. Animal preparation-Female SD rats weighing between 200-270 grams were anesthetized with sodium pentobarbital. The urinary bladder was catheterized with PE 50 tubing, sutured in places. The right external jugular vein was cannulated with PE 50 tubing, which was tunneled sub-cutaneously to exit at the mid-back. The rats were then placed in specially designed rat holders, which provided the necessary restraint without significant discomfort for the animal. The operative procedure was carried out, the night before the infusion experiment, and all animals were allowed a minimum of 14 hours recovery time.

2. 6 major and 2 minor groups were defined.

a) Group 1 - 20% expansion of ECV $\bar{c}$ 0.9% saline

b) Group 2 - 20% expansion of ECV $\bar{c}$ D_5/H_2O

c) Group 3 - 20% expansion of ECV $\bar{c}$ 0.9% saline then continued replacement of urine output to maintain saline diuresis.

d) Group 4 - 20% expansion of ECV $\bar{c}$ D_5/H_2O and continued replacement of urine output to maintain water diuresis.

e) Group 5 - 15% expansion of total body sodium (calculated as 45 meq/kg body wgt) $\bar{c}$ 3% saline.

f) Group 6 - Initiation of saline diuresis $\bar{c}$ 20%, replacement for 4 hours of urine output $\bar{c}$ 0.9% saline, then replacement of urine output for 5 hours $\bar{c}$ a solution of 0.9% saline and 25% bovine albumin (6.5 ml/kg).

g) Group 7 - 20% expansion with D_5/H_2O for one hr. then a 3 hour collection followed by 20% with NS and two hours of collection.

h) Group 8 - As in group 7 but with the order of D5 and NS reversed.

Groups 1-6 contained 8 animals per group, groups 7-8 each had 4 animals.

3. All urine samples were collected in 1 hour periods starting one hour preinfusion and continuing for 6 hours. Expansions and volume replacement took place over 1 hr. Separate aliquots of urine were prepared for sodium and potassium determination and kallikrein measurement. The samples for kallikrein were placed in tubes containing 0.025% sodium azide and all urine samples were quick frozen in dry ice and stored until evaluated.

4. Urinary Na^+ and K^+ were measured by flame photometry. Kallikrein was measured by both radioimmunoassay and a radiochemical technique.

5. Statistical analysis was performed with a special program for ANOVA for experiments with repeated measures, and a student T test.

Results:

1. The design of the experiment required that the animals be conscious. As rats are very susceptible to stress, holders were designed that provided the necessary restraint with maximum freedom. Rats kept in the holders for periods up to 24 hrs. demonstrated no excessive irritability.

2. 20% expansion of the extracellular space is equivalent to 4% expansion of total body weight. Expansion of this degree provides observable change in urinary volume and salt excretion. Total body water was assumed to be 66% of body weight while ECV was assumed to be 33% of total body water. A review of the literature supplied this information.

3. Evaluation of the raw data revealed significant intragroup variation. This is not unexpected in whole animal studies, and appropriate group size was used to allow legitimate statistical analysis. Base line equilibration periods were similar between groups.

4. Four factors were evaluated: Absolute amounts of Na, K, and kallikrein excreted and urinary volume. The following tables are mean values for each group.

In group 1, normal saline expansion alone produced similar but lesser magnitude changes as in group 3.

In group 2, D5W expansion alone produced similar but lesser magnitude changes as in group 4.

In group 3, normal saline diuresis produced persistent increases in urine volume and sodium excretion but only transient early increases in potassium or kallikrein excretion.

In group 4, D5W diuresis produced marked increases in urine volume but only minimal changes in sodium, potassium or kallikrein excretion.

The data on the 3%, NS-Albumin, and dual expansion groups are incomplete at this time.

5. Analysis of variance for the data revealed:

	Na	K	V	Kal
Group 1 NS	sig.	non-sig.	sig.	sig.
Group 2 D5W	non-sig.	sig.	sig.	sig.
Group 3 NSD	sig.	non-sig.	sig.	sig.
Group 4 D5D	non-sig.	sig.	sig.	non-sig.

Key: V=volume NS=normal saline D5=5% dextrose water Na=sodium K=potassium D=diuresis Kal=kallikrein

This table presents the results of ANOVA within each group. As can be noted, expansion provided the significant increase in urinary volume, and sodium loading significantly changed sodium excretion. The pattern of relationship between kallikrein excretion and the other variables is not completely clear at this point, i.e., Kallikrein changes with both sig. and non-sig. changes in urinary volume, sodium, and potassium. Further evaluation of trends, and completion of the additional groups will be necessary before final conclusions are drawn.

Significance of Findings:

The control of volume is crucial for an organism's survival. Control of salt and water excretion is central to volume regulation. The kallikrein-kinin system both alone and through connection to other renal mechanisms has been postulated to play a major role in this control. For the most part these conclusions are based on data provided from experiments in animal preparations not carefully standardized. The present experiments attempt to provide answers to basic questions. These are acute studies and are limited to relatively short term changes. However the degree of expansion is well within the range of normally tolerated physiologic changes, and has been demonstrated to significantly modify measured variables in the system. If no definitive relationship exists between kallikrein and volume control in the acute state, this does not preclude a role for the system in chronic adaptation to changes in salt and water metabolism.

Proposed Course:

1. To complete the groups noted above.

2. To study the effect of restraint in the holders on the measured variables.

3. To study the effect of acute potassium loading on kallikrein excretion.

4. To evaluate the role of the kallikrein-kinin system in renal "escape" from the effects of deoxycorticosterone. This will be done with chronic balance studies and kallikrein inhibition.

5. To apply more specific statistical methods to evaluate for relationships between Na, K, Vol, and kallikrein.

ANNUAL REPORT
SECTION ON BIOCHEMICAL PHARMACOLOGY
HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1979 to September 30, 1980

The research of our section represents a broad based approach to the understanding of neuronal systems. In the past year increasing attention has been devoted to protein phosphorylation, calcium-calmodulin mediated reactions and neuronal interactions as modulators of neurochemical systems. The primary direction of the work is toward understanding neural regulation of blood pressure although many of the studies have considerable relevance to neurological and mental illnesses.

I. Aminergic Systems

Catecholamines and serotonin serve as neurotransmitters in the central nervous system. The biosynthesis of these neurotransmitters in each case is regulated by the initial aromatic amino acid hydroxylase. These enzymes are tyrosine and tryptophan hydroxylase respectively. In prior years considerable effort has been devoted to characterizing these enzymes from both a molecular and regulatory point of view.

Tyrosine hydroxylase is a mixed function oxidase catalyzing the conversion of tyrosine to DOPA and requiring molecular oxygen and tetrahydrobiopterin cosubstrates. Work in previous years revealed that this enyzme has a molecular weight of 220,000 daltons and is composed of 4 apparently identical subunits. The enzyme appears to be regulated by phosphorylation-dephosphorylation reactions. In the phosphorylated form the enzyme has increased affinity for tetrahydrobiopterin, and decreased affinity of catecholamines which serve as end-product inhibitors and compete with the reduced pterin for a binding site on the enzyme.

Work has continued on this regulatory system and a modified view of its mechanism is emerging. Careful analysis of the pH profile of tyrosine hydroxylase indicated that the form normally isolated from brain had a pH optimum of about 5.8. Following protein phosphorylation this optimim shifted to pH 6.2. Kinetic analysis indicated that the Michaelis constant for the non-phosphorylated form of the enzyme with regard to tetrahydrobiopterin is pH dependent, increasing at pH values above 5.8. Conversely with the phosphorylated form the K_m for tetrahydrobiopterin (THB) was independent of pH. Clearly the regulatory system which has little impact on the enzyme at pH 5.8 is much more dramatic as physiologic pH is approached. This observation has permitted a further understanding of the regulatory process. Detailed kinetic analysis of either adrenal or brain tyrosine hydroxylase at pH 6.8 easily resolved the two kinetic forms. At this pH, the activated form had a K_m for THB of less than 10^{-5} M whereas the K_m for the inactive form was greater than 10^{-3}. Because the prevailing tissue concentration of THB is probably below 10^{-3} M, the phosphorylation of tyrosine hydroxylase at physiologic pH specifically switches the enzyme from an essentially inactive form to a highly active form. Analysis of the kinetic data by the Eadie-Hofstee method allows quantitation of each form of the enzyme. In rat striata and in adrenal medulla, it appears that about 20% of the total tyrosine hydroxylase is in the active form. If the enzyme is analyzed in adrenal medulla from rats that have been decapitated, there is a sharp increase in the percentage of the enzyme in the active form. This would suggest that regulation of tyrosine hydroxylase and consequently catecholamine synthesis is dynamic with significant changes in activity occurring within seconds.

Tryptophan hydroxylase is a distinct but similar enzyme and its activity in serotonin neurons accounts for the regulation of serotonin synthesis. This enzyme is less well characterized than tyrosine hydroxylase. Recent studies in our laboratory suggest it also has a molecular weight of about 220,000. The enzyme appears to be oxygen sensitive and is rapidly inactivated at 37° in an oxygen atmosphere. The activity of the enzyme can be largely recovered by incubating for 24 hours in the presence of thiols and ferrous ions. It appears that the enzyme is inactivated by sulfhydryl oxidation or exchange reactions. The recovery of activity is stimulated by inorganic sulfide and the enzyme is strongly inhibited by iron chelating substances suggesting the possibility that tryptophan hydroxylase may be an iron-sulfur protein. This conjecture cannot be resolved, however, until a system to purify the enzyme is devised. This is one of the continuing objectives of our laboratory. Tryptophan hydroxylase from brain can also be further activated by protein phosphorylating conditions. Previous work suggested that this reaction is calcium dependent. In the current year we have demonstrated that this activation is both Ca^{++} and calmodulin dependent. The development of an affinity column (vide infra) to quantitatively remove calmodulin from partially purified enzyme extract has been used to demonstrate absolute dependence on calmodulin for the ATP-Mg^{++}, Ca^{++}-induced activation process. Of particular interest is the observation that all the phenothiazine antipsychotic agents which are known to bind to calmodulin also inhibit the activation of tryptophan hydroxylase. These compounds are effective at μM concentrations. While the relevance of this observation to the mechanism of action of these drugs is unknown, it has been reported that patients receiving these drugs have reduced levels of the serotonin metabolite, 5HIAA in their cerebrospinal fluid.

Impulse flow and the associated release of neurotransmitter appears to be closely coupled to regulatory mechanisms for transmitter synthesis. Considerable evidence exists that during impulse flow there is a rapid influx of Ca^{++} that leads to the exocytotic release of transmitter stored in vesicles. A continuing line of experiments suggest that an alternate method of release may involve a Ca^{++}-dependent outward transport phenomenon. In this system norepinephrine stored in nerve terminals in heart slices appears to be released by an active transport mechanism which resembles the active reuptake system that normally transports the amine into the neuron following its exocytotic release. It has been observed that extracellular ATP inhibits this release process and detailed study of this inhibition suggests that both plasma membrane and membrane of storage vesicles contain an amine transport system and that prior to release these membranes coalesce to form a structure that retains the active transport system.

II. Role of Calcium and Calmodulin in Neurotransmitter Regulation.

It has long been recognized that calcium plays an important role in excitable tissues and in particular neurotransmission. In recent years the

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discovery of calmodulin, the protein which modulates many of the effects of calcium, has permitted increased understanding of the role of calcium. One of the major obstacles to studying calmodulin has been the lack of a sensitive and specific means of measuring this protein. In the previous year we reported the development of an enzyme linked immunoassay following the preparation of antisera to calmodulin. This assay has been used in the current studies on the mechanism regulating the distribution of calmodulin between membrane and cytosolic compartments. Brain tissue contains relatively large amounts of calmodulin, particularly in the synaptosomal membrane fraction (4 μg/mg protein). This strategic location of calmodulin suggest a role for it in neurotransmission and receptor mechanism. Studies on the manipulation of the dopamine receptor in corpus striatum have shown that activation of the dopamine receptor leads to a translocation of calmodulin from the membrane to the cytosolic fraction. Analysis of this soluble fraction indicates that calmodulin exists both as free calmodulin as well as being bound to the large molecular fraction. Presumably this latter portion is associated with cAMP phosphodiesterase and other calmodulin dependent soluble proteins. After receptor stimulation a greater proportion of the calmodulin is associated with the large molecular weight fraction. The fact that calmodulin has become bound to phosphodiesterase is reflected by an appropriate change in the kinetic properties of this enzyme. Thus it would appear that the dopamine receptor which is coupled to adenylate cyclase is also involved in the release of calmodulin from membrane stores. This in turn limits the action of the dopamine receptor by activating phosphodiesterase which catalyzes the hydrolysis of the cAMP.

The description of calmodulin dependent systems in crude tissue extracts requires a system for specifically and quantitatively removing calmodulin. The finding that many of the phenothiazines bind to calmodulin in a calcium dependent manner led to the development of an affinity chromatographic technique for purifying calmodulin. We have extended this approach by preparing fluphenazine coupled to sepharose. Calmodulin-free extracts can be prepared by chromatography on small pasteur pipette columns in the presence of calcium. This technique has proven extremely useful in identifying the nature of the protein kinase responsible for the activation of tryptophan hydroxylase (*vide supra*) and in our studies on the calcium dependent phosphorylation of other endogenous proteins. It is anticipated that this technique will attain wide use for the identification of calmodulin dependent-reactions. While it remains to be determined whether a calcium-calmodulin dependent kinase influences catecholamine synthesis, synaptosomal studies suggest that calcium can stimulate tyrosine hydroxylation in cortical synaptosomes. Since Ca^{++} does not affect tyrosine uptake it is possible that calcium is acting through a protein kinase system. It is clear that calmodulin and calcium play key roles in the regulation of neurotransmission and work in our laboratory increasingly focuses on this second messenger system.

III. Protein Phosphorylation in Neuronal Systems

From the above discussions it is obvious that considerable effort in our group is directed toward the role of protein phosphorylation in neuronal systems. During the last two years we have intiated research on the phosphorylation of endogenous proteins and the corresponding kinases. We have observed that synaptosomal membranes from brain incorporate phosphate from ATP in the presence of Mg^{++} into a number of specific membrane proteins in a

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calcium dependent manner. This pattern of protein phosphorylation is altered in morphine-addicted animals. More recently we studied the endogenous phosphorylating system in synaptosomal cytosol. These extracts show little phosphorylation in the presence of EGTA. Adding cAMP to the system caused about a two-fold stimulation. Calcium, however, stimulates PO_4 incorporation about 25-fold. This dramatic calcium stimulation is apparent in a number of substrates. However, it is most marked with protein substrates whose molecular weight are 50, 55 and 60K. As little as μM concentrations of calcium stimulate phosphate incorporation. These phosphorylations were powerfully inhibited by the anti-psychotic fluphenazine suggesting that this cytosolic kinase (s) is calmodulin dependent. Passage of these extracts through fluphenazine-sepharose columns rendered them unresponsive to calcium without the addition of calmodulin, which restored full activity.

In the course of studying the membrane phosphorylation system we examined the effects of heated extracts upon phosphorylation. In addition to the calmodulin activity, the heated extracts were found to contain another heat stable factor which could stimulate the phosphorylation of a specific protein in a calcium independent manner. We are currently attempting to isolate and characterize this regulatory factor.

As discussed above there are protein kinases in brain that require calmodulin and calcium. Other laboratories have reported that pro-protein kinase exists in brain and that this prokinase can be activated by a calcium dependent protease. We isolated this prokinase in pure form and found that the active kinase generated from this protein is itself stimulated by calcium and may have calmodulin bound to it. The role of the enzyme generated from this prokinase is under continuing study.

IV. Tetrahydrobiopterin (THB), The Hydroxylase Cofactor

The mechanism by which protein phosphorylation interacts with catecholamine synthesis is by changing the relative affinity of the hydroxylase cofactor and the end-product inhibitor for tyrosine hydroxylase. Since it would appear that the concentration of THB within many neurons may be rate-limiting for this reaction, knowledge of the concentration, biosynthesis, and regulation of the concentration of THB becomes very important in understanding these transmitter systems. In the past two years we have developed a sensitive assay for hydroxylase cofactor activity and in the past year applied to it several clinical and biochemical questions.

Analysis of THB has presented numerous problems, since the compound is present in very low concentrations in most tissue and is readily autooxidized. In the previous year we described a sensitive enzymic assay in which pure liver phenylalanine hydroxylase was used in the presence of a hydroxylase cofactor reducing system. In the current year an extremely sensitive HPLC system with fluorescence detection of THB has been developed. This system measures both oxidized and reduced THB and will allow better quantitation of the whole system.

While the content of hydroxylase cofactor in any brain region appears to be relatively low, the distribution suggests that it may be associated primarily with aminergic systems. Indeed, if the cofactor is only present in aminergic neurons the actual concentration within a nerve terminal would be considerably higher and consequently would have a significant impact on how its role in regulation is viewed. In the corpus striatum the vast majority of the aminergic terminals arise from dopamine cell bodies in the substantia nigra. Current experiments have shown that destruction of the nigral-striatal pathway results in an 80-90% loss of hydroxylase cofactor in the striatum. This important observation indicates that indeed THB is specifically contained in aminergic cells of the CNS. Since the dopamine terminals of the striatum occupy only a small proportion of the tissue mass the actual concentration within the dopamine terminal may be considerably higher than previously thought. These findings would complicate our understanding of how tyrosine hydroxylase is regulated were it not for our findings reported above suggesting that this enzyme is essentially inactive at physiological pH unless it is in the phosphorylated state.

The concept that the tissue concentration of THB may be important in the central synthesis of biogenic amines led us to develop systems to evaluate this key compound in man. Systems for measuring hydroxylase cofactor in cerebrospinal fluid (CSF) have been reported in prior years. Attempts to correlate this parameter with CNS amine metabolism have been partially successful. There appears to be a strong correlation between THB and homovanillic acid, the major dopamine metabolite in CSF. Other findings are a significant inverse relationship with age and the observation that hydroxylase cofactor activity is significantly reduced both in patients with Parkinson's disease and in certain individuals with familiar dystonia. These findings have led to the suggestion that administration of THB to patients with these neurological diseases may be helpful in alleviating some of the symptoms associated with the disease. Such studies are planned. Examination of a number of patients with affective disorders or schizophrenia have not revealed any abnormalities in CSF hydroxylase cofactor activity.

V. Post-Syanptic Regulation.

Work has continued on N-acetyl transferase in the pineal gland as a model system for how cells respond to neurotransmitter stimulation. In this system (either in vivo or in organ culture) pinealocytes respond to β-receptor stimulation by increasing the amount and activity of serotonin N-acetyltransferase (NAT). In vivo the enzyme activity shows a marked diurnal rhythm resulting from changes in nerve impulses arriving at the gland which in turn are controlled by light and darkness. Previously we reported that α-receptors in the CNS appear to be involved in mediating the light-dark response: α-receptor blocker delays the fall in enzyme activity in dark adapted animals exposed to light; conversely, an α-agonist precipitates a fall in enzyme activity even during darkness. In recent studies it has been observed that phenoxybenzamine can of itself cause an induction of the enzyme both in vivo and in organ culture. Not only does α-blockade cause an induction of NAT, but it also potentiates the well known induction resulting from β-agonist stimulation. Transient stimulation of cAMP content appears to be associated with the induction of NAT. It was, however, of interest to examine the effects of light on the cAMP content of the pineal gland in a dark-adapted rat. Interestingly while light causes an immediate drop in cAMP content by 2 min the cAMP content has returned prelight levels and by 7 minutes it is increased 2 to 3 fold. This would indicate that if a loss of cAMP is involved in the rapid reduction of NAT activity, then a reduction in cAMP for only a few seconds is sufficient to cause the decline in activity.

In a completely separate project the mechanism by which the carotid body responds to changes in oxygen tension is under continuing study. In prior years it was reported that hypoxia causes a release of dopamine from the glomus cells in the carotid and that 24 hours after a hypoxic episode there is an increase in tyrosine hydroxylase in these cells. The type of receptors mediating this response are unknown. In recent experiments it appears that stimulation of muscarinic receptors mimics the response seen following hypoxia. Also of interest is the observation that carbohydrate active steroids can also cause an induction of the dopamine synthetic machinery in the carotid. It is clear, however, that dopamine release within the carotid body plays an important role in the organ's response to hypoxia.

VI. Substance P.

Substance P is a neuropeptide that appears to serve as a neurotransmitter in several CNS systems. It has also been suggested to serve as a possible primary afferent neurotransmitter in the baro-reflex. In the past year we initiated work to study its synthesis and metabolism. Since peptides are both synthesized and degraded by proteases, enzyme inhibitors to specifically study these processes are not readily available and can lead to confusing results. It appears that compounds that were designed as angiotensin converting enzyme inhibitors are also effective inhibitors of Substance P metabolism. When these compounds are administered directly into the brain the content of Substance P increases by factor of 2. This is particularly interesting since these compounds are excellent antihypertensive agents, and their effects do not seem to be totally related to the degree of converting enzyme inhibition. Thus it may be possible that Substance P may also have a role in blood pressure regulation.

A more well defined role for Substance P neurons has been proposed for the nigrostriatal dopamine pathway. Here Substance P cell bodies reside in the caudate and send projections to the substantia nigra where it is an excitatory transmitter and serves a positive feedback role. Long term dopamine receptor blockade apparently causes a marked increase in the activity of this neuronal system and causes a significant decrease in Substance P levels probably reflecting an increased utilization (release). The levels of substance P "rebounded" beyond control levels 3 or 4 days following the cessation of dopamine receptor blockade suggesting that enhanced synthetic capacity had been stimulated during the period of increased nerve activity. Conversely, a dopamine receptor agonist caused an acute decrease in Substance P levels. We suggest that this may be due to an accumulation of transmitter following cessation of impulse flow. In any case it is clear that Substance P is intimately involved in the regulation of the nigrostriatal dopamine pathway.

VII. Blood Pressure Regulation

Central regulation of blood pressure continues to be one primary interest of our laboratory. In a new initiative we began an investigation of the role of central serotonergic systems in blood pressure regulation . Stimulation of serotonin neurons in the dorsal and median raphe results in an immediate and marked pressor response. This response was identified as being due to serotonin neuron stimulation both histologically and by a variety of pharmacological approaches. The circuitry for the response is not known but there is evidence that it proceeds rostrally and is integrated at the level of the hypothalamus. We are only beginning to understand the number and complexity of the neuronal systems in brain that regulate and modulate blood pressure. Such an understanding is a continuing long term objective of our laboratory.

Norepinephrine systems in the brain have long been suspected of being involved in blood pressure regulation and several years ago we postulated that there may be an inverse correlation between the activity of norepinephrine neurons and blood pressure. During the past year we have observed that one can increase the rate of norepinephrine synthesis by intraventricular injection of the precursor tyrosine. As little as 15 μg of the amino acid injected by this route is also effective in reducing the blood pressure in the genetically hypertensive rats. These data provide additional support for our concept concerning central norepinephrine and blood pressure.

Another type of neuron which are reported to be involved in blood pressure regulation are the epinephrine containing neurons. Genetically hypertensive rats are known to have increased amounts of phenylethanolamine N methyl transferase (PNMT) in their brainstem. In a separate study it was observed by immunochemical techniques that this increased enzyme activity is attributable to an increased number of cells rather than an increased amount of enzyme per cell. The genetic factors and mechanisms determining the number of specific cell types in brain is a subject that requires much additional study.

Another focus of work on hypertensive disease has been the interaction of genetic and environmental factors. In the previous report we found that the stroke-prone substrain of the spontaneously hypertensive rats (SHR), which experience about 80% incidence of stroke by 10 months of age when fed a typical Japanese rat chow, had less than 20% incidence of stroke when raised on the NIH rat chow. The only significant difference between these diets appears to be the protein content (Japanese, 17% and NIH, 24%). This initial study was done on a cooperative basis in Japan. During the past year we have repeated this study in our laboratory and have obtained similar results. While the sodium content of the two diets are similar, the higher protein content appears to stimulate the secretion of sodium. It is clear that the effect of increased protein intake goes beyond its naturetic properties since the severity of hypertension is similar on either diet. It is possible that the effect is on vascular structure. We previously reported that the rate of amino acid incorporation into protein is much greater in the mesenteric and spermatic arteries of the SHR than in normotensive animals. We are currently establishing methods for measuring amino acid incorporation in cerebral vessels and we plan to investigate the effect of dietary protein on the cerebral vessels. These studies however provide another example of the interaction of genetic and environmental factors in the pathogenesis of cardiovascular disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01843-06 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mechanisms of Storage, Uptake and Release of Norepinephrine in Adrenergic Nerve Endings (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Donald F. Bogdanski	Pharmacologist	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Evidence is presented to substantiate and more precisely define the postulate that catecholamine neucoltransmitters may be released by a neuronal transport process distinct from exocytosis. This is a continuing investigation of a model synaptic system consisting of a calcium dependent release of ^{3}H-norepinephrine (^{3}H-NE) from rat heart slices incubated in a Na$^+$ deficient medium. It appears that a calcium dependent process directs norepinephrine loaded intraneuronal binding sites to a transport site on the plasma membrane. The ^{3}H-NE appears to be passed directly from its binding site to the outward transport system. Amines therefore can be transported across an intact membrane without involving a process of exocytosis. Reserpine and Li$^+$ appears to disrupt this process since they elicit a calcium independent release of deaminated metabolites. Studies now indicate that ^{3}H-NE is transported directly from vesicles whose membrane to form a new structure that retained the transport mechanism of both. Thus the release was inhibited by extracellular, energy-conserving, phosphorylated nucleotides. A Mg++-ATP dependent transport mechanism in the membrane of storage vesicles has been reported.

Objectives: To study the effects of ATP and other nucleotides on the Ca^{++} dependent release of ^{3}H-NE in rat heart ventricle slices incubated in Na^+ deprived (choline+) media.

Methods: Rat hearts were labelled with ^{3}H-NE in vivo and then ventricle slices were prepared and incubated as described in earlier reports. The medium was sampled for the determination of the total radioactivity to be used for calculating the rate of release of ^{3}H-compounds. For experiments used to test the effects of ATP on the release of tissue ^{3}H-NE, the experiments were modified slightly as follows. After the standard preliminary incubation of slices in KRBS, the slices were transferred to standard Na^+ deficient (choline - Ca^{++}) medium with 12.5 mM $NaHCO_3$. The slices were incubated and the media sampled every 20 min for 60 min. The slices were then transferred to fresh Na^+ deficient (choline-Ca^{++}) medium containing 3 mM ATP and, either 0, 1.18 or 2.36 mM Mg^{++}. The pH was adjusted to 7.3. The slices were incubated an additional 80 min and the media was sampled every 20 min. For other experiments, tissues were dialyzed as described in previous reports. Rats were injected with 1-^{3}H-NE, killed 18 hours later, and heart slices were incubated 90 min in KRBS. The slices were immersed in ice cold modified KRBS containing 1% of the usual Ca^{++} and Mg^{++} and 10% of the dextrose. Other dialysis solutions contained either Li^+, K^+ or choline in place of Na^+. After 18 hrs, the slices were incubated in various media as described in the "Results."

Major Findings: The results of this series of experiments is summarized as follows:

(1) Extracellular ATP, at a concentration of 3 mM, had no effect on the release of ^{3}H-compounds in slices incubated in KRBS.

(2) Extracellular ATP moderately inhibited the Ch^+-Ca^{++} stimulated release.

The interpretations of results (1) and (2) are based upon previous results showing that different processes are involved in releasing ^{3}H-compounds in the two media. Deaminated metabolites are released in KRBS, whereas amines are the predominant compounds released by Ch^+-Ca^{++} stimulation. Since transport inhibitors block Ch^+-Ca^{++} stimulated release, it is concluded that release of amines is mediated by transport of the amines through a diffusion barrier. The results of previous experiments reported in the previous years indicate that the amines are released from intact vesicles located in close proximity to the transport mechanism at the plasma membrane. The ^{3}H-NE released from these vesicles also can be controlled by ATP in the extracellular medium. Possible actions of the nucleotide are: stimulation of the uptake of released ^{3}H-amine, inhibition of outward transport (by analogy with the transport inhibitors, cocaine and desipramine), or inhibit ion of the Mg^{++}-ATP stimulated transport mechanism in the membrane of the vesicles.

(3) The uptake of ^{3}H-NE by slices incubated in KRBS was neither stimulated nor blocked by ATP, unlike cocaine, which blocked transport in parallel experiments. Therefore, ATP does not block transport through the plasma membrane by an action analogous to that of cocaine. An action upon the membrane of the vesicle appears to be a more likely site of the ATP action.

(4) The inhibition of release by extracellular ATP was facilitated by 2.5 mM Mg^{++}. Other nucleotides, including ADP, GTP and UTP, also inhibited ^{3}H-NE release. These effects were similar to those reported by Euler for isolated, bovine splenic nerve vesicles. It is tentatively concluded that extracellular, phosphorylated, energy conserving nucleotides have access to the membrane of vesicles in adrenergic neurons incubated in Na^{+}-deprived ($choline^{+}$-Ca^{++}) media.

The next series of experiments was performed to study this problem. Attempts were made to study the effects of ATP upon neuronal vesicles which were not attached to the plasma membrane transport mechanisms. The effects of ATP as these vesicles were compared with the effects of extracellular ATP on Ch^{+}-Ca^{++} stimulated release.

The ATP was presented to the tissues by the process of dialysis, with the cell membrane acting as an dialysing membrane. The tissues and dialysing medium were kept at 0° for 18 hours. During this time, the extracellular ATP either permeated the cell membrane or penetrated to a specific site in the membrane. The results of this series of experiments follows:

(1) Unlike non-dialyzed ATP, the dialyzed ATP inhibited the release of ^{3}H-compounds in tissues subsequently incubated in KRBS.

(2) Cells dialyzed without ATP released more ^{3}H-compounds when incubated in KRBS containing cocaine. The evidence thus indicated that the release of ^{3}H-NE was controlled by a pump and leak system. Inhibition of the pump prevented the uptake of leaked ^{3}H-NE, thus increasing the quantity of ^{3}H-NE entering the incubation medium.

(3) Dialyzed ATP inhibited the release of ^{3}H-NE in slices incubated in KRBS containing cocaine. The ATP inhibited the leakage of ^{3}H-NE in the tissue into the extracellular fluid with the result that cocaine could not stimulate release to the medium.

(4) Dialyzed ATP inhibited the release of ^{3}H-NE during the first 60 min of incubation of slices in the Ch^{+}-Ca^{++} medium. Previous studies had shown that, without the ATP, the release of ^{3}H-NE during this time was not Ca^{++} dependent, nor strongly inhibited by cocaine. Thus, the release was not Ch^{+}-Ca^{++} stimulated release, but was analogous to the spontaneous release of ^{3}H-compounds such as that which occurs in slices incubated in KRBS. After 60 min, the release from slices dialyzed with ATP paralleled that from slices dialyzed without ATP.

(5) After 60 min, the release is Ca^{++} dependent and can be inhibited by cocaine. Thus, the release characteristics conform to those of Ch^{+}-Ca^{++} stimulated, transport-mediated release. Moreover, this release was inhibited by ATP in the incubation medium. The results of the experiment indicated that after the vesicles became oriented to the transport sites in the plasma membrane, the vesicles were accessible to extracellular ATP, and less accessible to dialyzed ATP.

(6) Dialyzed ATP inhibited the release of ^{3}H-NE in Ch^+-Ca^{++} stimulated slices which had been dialyzed with Na^+, Li^+, K^+, or Ch^+. However, cocaine elicited an additional increment of inhibition only in those slices dialyzed with the Na^+ dialysis solution, but not any of the other solutions. Thus, intracellular Na^+ would appear to be required to orient the vesicles to the amine pump in the plasma membrane in Ch^+-Ca^{++} stimulated slices.

(7) The results of this series of experiments are in accord with the view that the vesicles retain their stores of ^{3}H-NE when exposed to dialyzed ATP. After orientation to the amine pump, these vesicles are accessible to extracellular ATP. The effect of either dialyzed ATP or extracellular ATP on slices are similar to the effects of ATP on isolated splenic nerve vesicles. The conclusions, drawn from the above results of experiments is that the vesicle membrane coalesces with the plasma membrane to form a new structure which retains the transport mechanisms in both.

Significance to Biomedical Research and Institute Programs: The possible significance of these studies to an understanding of basic physiological processes is the fact that there is increasing evidence that exocytosis is not the only mechanism of secretion. The release processes in slices incubated in Na^+-deficient media (choline$^+$) containing Ca^{++} show numerous parallels to neurotransmission as discussed in earlier reports. Hence, our findings are relevant to neurosecretion and synaptic transmission. Our conclusions outlined above indicate the existence of specific Ca^{++}-dependent processes for transporting binding sites to the plasma membrane and suggest that mobilization and transport of NE may be intimately associated and possibly are mediated by the same mechanism. The evidence now suggests that the vesicle and plasma membranes coalesce to form a new structure which retains the NE transporting mechanisms of both membranes. During the process of coalescence, vesicle membrane DBH and ATP could be released to the extracellular medium.

Proposed Course of Project: The basic structure of the model neurosecretory system is now postulated and many details of the mechanisms involved fit available data. Such details as can be verified experimentally will be sought.

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PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Biology to Chemoreceptor Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	Sten Hellstrom	Visiting Scientist	HE NHLBI
	Farouk Karoum	Pharmacologist	LCP NIMH
	Sukhamay Lahiri	Professor	University of Pennsylvania

COOPERATING UNITS (if any)
Laboratory of Clinical Pharmacology, NIMH, St. Elizabeth's Hospital, Washington, D.C., and Institute of Environmental Medicine, Univ. of Pennsylvania, Philadelphia, Pennsylvania

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The mechanisms whereby the catecholamine content of carotid bodies is regulated by physiologic or pharmacologic stimuli was studied. Short-lasting exposure to hypoxia and stimulation of muscarinic receptors increases the rate of dopamine release without changing its turnover-rate. Neither the carotid sinus nor the sympathetic nerves participate in the modification of dopamine content by both types of stimuli. Injection of carbohydrate-active steroids causes a transient increase of dopamine content due to blockade of dopamine metabolism and a long-lasting increase in norepinephrine content which depended on an increased protein synthesis.

Objectives: The carotid body is a chemoreceptor organ which senses changes in the partial pressure of arterial blood gases. In mammals the regulation of the acute response to hypoxia is triggered by stimulation of chemoreceptors located in the carotid body. During long-lasting hypoxia compensatory changes in circulation, rate of respiration and pulmonary gas exchange are greatly reduced in the absence of carotid body chemoreceptors. The glomus cell is the most prevalent cell type in the carotid body and may be involved in the control of chemoreceptor afferent discharge. Dopamine was shown to be the major neurotransmitter in these cells, whereas norepinephrine is primarily located in sympathetic nerve endings of the carotid body vasculature. It is of interest to determine whether in carotid body the catecholamine content of glomus cells or of the perivascular sympathetic nerve endings is affected by physiological or pharmacological stimuli.

Methods: The concentrations of dopamine, norepinephrine, dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by gas chromatography-mass fragmentography. The carotid bodies were homogenized in 0.1 N formic acid and deuterated analoges of the catecholamines and metabolites were used as internal standards. The turnover-rate of dopamine was determined by measuring the initial decline of the DOPAC content after injection of pargyline.

In some studies transection of the carotid sinus serve, or superior cervical ganglionectomy was performed unilaterally 5 to 7 days before the experiment.

Major Findings: Effect of methacholine on dopamine storage in carotid body. Injection of methacholine (12.5 μmol/kg) decreases the dopamine content of rat carotid body without changing the norepinephrine content. This decrease in dopamine content is due to an increased rate of release of this neurotransmitter and also occurs after transection of the carotid sinus nerve or ganglionectomy. Methylatropine (21 μmol/kg) prevents the decrease of dopamine content elicited by methacholine. Since methylatropine could block also the decrease of dopamine content elicited by short-term hypoxia it is inferred that a muscarinic receptor may play an important role in the mechanism, whereby the carotid body senses changes in arterial blood gases. In fact, the release of dopamine elicited by exposure to hypoxia can be curtailed by atropine.

Increase of dopamine and norepinephrine content by carbohydrate-active steroids. Short-term or long-term administration of dexamethasone (1 mg/kg,i.p.) increases the concentrations of dopamine and norepinephrine in rat carotid body. This increase occurs also after transection of the carotid sinus nerve or ganglionectomy. Injections of cycloheximide curtail only the increase in noradrenaline content but not the increase in dopamine content.

Studies on the turnover of dopamine in carotid body revealed that the rate of elimination of DOPAC was reduced more than 50% 12 hours following the injection of dexamethasone, while the steady content of dopamine is increased. From these results it was concluded that the regulation of dopamine and norepinephrine content by dexamethasone underlies two different mechanisms. The increase of dopamine content is due to a reduction in its metabolism,

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whereas the long-term increase of norepinephrine appears to be triggered by a process, which involves new protein synthesis.

Increase of dopamine and norepinephrine content by long-lasting exposure to hypoxia. Exposure to hypoxia lasting for 2 days increases the dopamine content in rat carotid body, but fails to change that of norepinephrine. Exposure to hypoxia lasting one week increases the content of both dopamine and norepinephrine. The levels of both amines increase even further, when the exposure time to hypoxia is extended to 2 and 4 weeks. Denervation of the carotid body fails to prevent this increase. The specificity of these changes in carotid body catecholamine content is upheld by the finding that exposure to hypoxia for 4 weeks fails to change the catecholamine content in caudate nucleus, heart, superior cervical ganglia and adrenal glands.

Significance to Biomedical Research and Institute Program: The arterial chemoreceptors play an important role in the respiratory and circulatory response to hypoxia, hypercapria or metabolic acidosis. One of the main problems in carotid body function is to understand the mechanism whereby chemosensory impulses are initiated. To analyze physiologically and biochemically the events occurring at the receptor level, it is necessary to know how the carotid body is structurally organized and whether there are specific neurotransmitters involved in the chemosensory response. Our studies reveal that dopamine present in the glomus cell is involved in the chemoreceptor response during short- and long-lasting exposure to hypoxia.

Proposed Course of Project: The following experimental approach is planned to improve our understanding of the biochemical mechanisms involved in chemoreceptor function.

(1) Studies on the effect of hypercapnia and metabolic acidosis on the dopamine and norepinephrine content of rat carotid bodies.

(2) Measurement of the peptide hormones - metenkephaline and vasoactive intestinal peptide - in carotid bodies of dogs during exposure to hypoxia or hypercapnia.

Publications:

1. Hellstrom, S., Commissiong, J. and Hanbauer, I.: Modification of the dopamine and noradrenaline content in rat carotid body by carbohydrate-active steroids. Neuroscience 4:1157-1162, 1979.

2. Hellstrom, S. and Hanbauer, I.: Modification of the dopamine content in rat carotid body by methacholine and hypoxia. Proceedings of the Carotid Body Workshop in Vallidoloid, Spain (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01847-06 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Functional Role of Calmodulin in the Regulation of Neurotransmitter Receptors (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Sikta Pradhan	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Persistent stimulation of striatal dopamine receptors increases the calmodulin content in the supernatant fraction. This increase appears to be triggered by membrane phosphorylation catalyzed by a cAMP-dependent protein-kinase. In the supernatant fraction prepared from striatal slices cAMP-phosphodiesterase has a biphasic kinetic profile. Incubation of striatal slices with dopamine or opiate receptor agonists changes the biphasic profile to a monophasic function. Haloperidol prevents the changes elicited by both types of agonists. In addition, the changes in PDE kinetic properties and calmodulin content elicited by morphine were also curtailed by naltroxone and striatal deafferentation.

PHS-6040
(Rev. 10-76)

Objectives: Since in the CNS a complex system of heterogenous inter-connected neurons and associated neuroglia is operative, the participation of cyclic-nucleotides in the synaptic transmission must be studied along tactical lines. The goal of this study was to obtain information in the molecular mechanisms underlying the stimulation of dopamine receptors. There exists ample evidence for a direct relationship between dopamine receptors and the presence of dopamine-sensitive adenylate cyclase in caudate nucleus. It is well established that the cyclic nucleotid content is regulated by nucleotide cyclases and cyclic nucleotide phosphodiesterase. Both enzymes are regulated by a common Ca^{2+}-dependent regulator protein called calmodulin.

The present experiments were carried out to evaluate the participation of calmodulin in the function of dopamine receptors. It was of interest to establish how the compartmentation of calmodulin and the apparent kinetic properties of PDE can be changed during persistent stimulation of dopamine receptors.

Methods: Rat caudate nuclei slices were incubated in Krebs-Ringer solution pH 7.4 supplemented with ascorbic acid and dextrose. After preincubation for 60 min the drug under study was added and the incubation was continued for 30 min. The slices were then drained and homogenized in 0.32 M sucrose. After centrifugation at 4000 rpm for 10 min the supernatant was removed and recentrifuged at 39.000 rmp for 30 min. The pellet-fraction was extracted with Tris-HCl buffer pH 7.4 containing 0.1% lubrol.

The kinetic properties of cAMP-phosphodiesterase were studied in the supernatant fraction of striatal homogenates. The content of calmodulin was estimated by micro-ELISA in both supernatant fraction and pellet-extract prepared from striatal homogenates. Hemitransection of the nigra-striatal fibre bundle was performed 3 weeks before the experiment.

Major Findings: 1. Changes in striatal calmodulin content elicited by stimulation of dopamine receptors. Incubation of striatal slices with apomorphine (10^{-7} M) or dopamine (2×10^{-7} M) increases the calmodulin content in the supernatant fraction prepared from striatal homogenates. The amount of calmodulin associated with cAMP-PDE is increased after persistent stimulation of dopamine receptors. Similarly, incubation of striatal slices with morphone (10^{-6} M) increases the calmodulin content in the supernatant fraction and also the amount of calmodulin associated with cAMP-PDE. The central acting stimulant d-amphetamine was also tested. Since it was shown to increase the dopamine turnover and to elicit a transient increase in striatal cAMP content. d-Amphetamine injected intravenously 45 min before killing of the rats increases the calmodulin content in the supernatant fraction prepared from striata.

2. Changes in the kinetic properties of cAMP-PDE in striatal slices. In control conditions cAMP-PDE in striatal slices exists in at least two kinetic forms, one with a low and one with a high K_m for cAMP. After incubation with dopamine receptor agonists the biphasic double reciprocal plot of initial velocity versus cAMP contentration appears as a monophasic one.

Haloperidol (10^{-7}M) prevents this change. Transection of the nigra-striatal fibre bundle by itself does not change the kinetic profile of striatal cAMP-PDE. In slices prepared from deafferented striata activation of dopamine receptors still elicits a decrease in the K_m for cAMP indicating that calmodulin and the calmodulin activatable cAMP-PDE are located in post-synaptic neurons.

Incubation of striatal slices with morphine (10^{-6}M) lowers the K_m for cAMP and changes the kinetic profile from biphasic to monophasic. These changes in the apparent K_m for cAMP elicited by morphine can be blocked by haloperidol (10^{-7}M) and by naltrexon (10^{-7}M). In slices prepared from deafferented caudate nuclei morphine (10^{-6}M) fails to cause a change in the kinetic properties of cAMP-PDE. Injection of d-amphetamine elicits a decrease in K_m for cAMP of striatal.

Significance to Biomedical Research and Institute Programs: Considerable attention has been paid to cyclic nucleotides and their regulation in various tissues and various pathological states. Since cAMP participates as a second messenger linking receptor activity to biochemical processes, our studies on the response to dopamine receptor stimulation are of interest for the clarification of the action mechanism of neuroleptic and narcotic drugs.

Proposed Course of Project: The functional role of second messengers on the regulation of neurotransmitter responses will be extended to other neuronal systems, which form synaptic interconnections with the striatal dopaminergic neurons. The input on dopamine receptor activation by glutaminergic or gabaminergic neurons will be studied using calmodulin, adenylate cyclase and cAMP-PDE as biochemical indices.

Publications:

1. Hanbauer, I., Gimble, J. and Lovenberg, W.: Changes in soluble calmodulin following activation of dopamine receptors in rat striatal slices. Neuropharmacology, 18: 851-857 (1979).

2. Hanbauer, I., Gimble, J., Sankaran, K. and Sherard, R.: Modulation of striatal cyclic nucleotide phosphodiesterase by calmodulin: Regulation by opiate and dopamine receptors. Neuropharmacology 18: 859-864 (1979).

3. Hanbauer, I.: Calmodulin in dopamine receptor function. In: Psychopharmacology and Biochemistry of Neurotransmitter Receptors (Olson, R, Usdin, E. and Yamamura, H., eds.), M. Sekker, Philadelphia (In press).

4. Hanbauer, I. and Costa, E.: Role of calmodulin in dopaminergic transmission. In: Calcium and Cell Function Calmodulin (Cheung, W.Y., ed), Vol. 1, Acadmiec Press, New York (In press).

5. Hanbauer, I.: Participation of calmodulin in the regulation of dopamine receptors. In: Catecholamine Basic and Clinical Frontiers. Usdin, E., Kopin, I.J. and Barchase, J., eds., Pergamon Press, N.Y., 1979, pp. 1212-1214.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01850-11 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biochemistry of the Spontaneously Hypertensive Rat

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Dr. M. Fujiwara	Visiting Fellow	HE NHLBI
OTHER:	Dr. Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Dr. Yukio Yamori	Guest Worker	HE NHLBI
	Dr. Ryochi Horie	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.8	OTHER: 0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The role of protein metabolism in the vasculature has been investigated with regard to the pathogenesis of hypertension and stroke in genetic animal models. Based on our previous observation that incorporation of amino acids into vascular proteins in young spontaneously hypertensive rats was significantly increased, we attempted to set up a system for measuring amino acid incorporation into brain microvessels. This has been successful. Nutritional studies have demonstrated that the incidence of stroke in the stroke-prone substrain of the gentically hypertensive is related to the protein intake. This would suggest a possible correlation between dietary protein and vascular structure although this remains to be determined.

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Objectives: Vascular protein synthesis and wall structure may be important parameters in the pathogenesis of stroke and hypertension. In prior years we had shown that the rate of amino acid incorporation into peripheral vascular protein of hypertensive animals is increased. The object of work in the current year is to determine whether cerebral vessels isolated by biochemical techniques demonstrate the same phenomenon and whether this vascular protein synthesis in any way relates to the incidence of stroke in the genetically stroke-prone rats. We also wished to determine the effect of dietary protein on the incidence of stroke in stroke-prone animals.

Methods: Cerebral microvessels were isolated by modifications of established techniques. Incorporation of radioactive lysine into the protein of these vessels was measured 2 hours after a pulse injection. Blood pressures were monitored by tact-cuff plethysmography. For nutritional experiments animals were obtained either from NIH small animal section or the Stroke-Prevention Center in Izumo, Japan. Starting at 6 weeks of age animals were maintained in randomly divided groups for up to 10 months of age. At this age animals which had not succumbed to stroke were sacrificed and their brains examined histologically.

Major Findings: Substantial effort was devoted developing a system for the clean separation of brain microvessels. The final proceudre gave relatively pure preparations of vascular material. We next attempted to measure the rate of amino acid incorporation. The incorporation of amino acids was easily measured in both the collagen and non-collagen protein of the preparation. In fact the rate of incorporation is significantly greater than that seen in mesentric vessels. We are currently determining whether the difference seen previously in the peripheral vessels of the spontaneously hypertensive rats is also seen in the cerebral microvessels. Comparison of amino acid incorporation in the brain microvessels in stroke-prone animals will be done since it may be weakness in these vessels that leads to stroke.

In the previous year we reported a cooperative study done in Japan in which stroke-prone rats from either the NIH or the Izumo colony were maintained during the 1st 10 months of life on either Japanese rat chow or NIH rat chow. The striking finding was that animals maintained on the NIH diet had almost no stroke whereas 60% of the animals on the Japanese diet had neurological symptoms of stroke and 80% had cerebral lesions upon autopsy. The only major difference in the two diets appeared to be the protein content (NIH, 24%; Japanese, 17%). During the past year we have repeated this experiment in our laboratory. Male stroke-prone spontaneously hypertensive rats were divided into 2 groups of 35 each at about 5 weeks of age and maintained either Japanese or NIH rat chow. By 9 months of age the mean body weight of the two groups was identical 305 gm. Blood pressures were slightly higher in the animals receiving the NIH rat chow (233 ± 9 mm Hg vs 211 ± 3 mm Hg). Histological examination of the brain indicated 80% incidence of stroke on the Japanese and less than 20% on the NIH diet. This confirms our previous experiment and suggests that the amount and quality of protein in the diet can effect the incidence of stroke.

Significance to Biomedical Research and Institute Programs: The identification of risk factors associated with cardiovascular disease is one of the prime objectives of the NHLBI. Based on our studies with the stroke-prone spontaneously hypertensive rat it would appear that while hypertension is one of the major risk factors for stroke, this factor appears to be modified by the dietary protein intake. If these findings can be extended to man, a preliminary epidemiologic study in Japan supports this effect, than our view on the prevention of stroke will be altered.

Proposed Course of Project: In the coming year we plan to examine the effect of dietary protein on the rate of protein synthesis in cerebral microvessels.

Publications:

1. Yamori, Y., Horie, R., Ikeda, K., Nara, Y. and Lovenberg, W.: Prophylactic effect of dietary protein on stroke and its mechanisms. In: Perspectives in Cardiovascular Research, Arnold M. Katz (Series ed.), Vol. 4, Prophylactic Approach to Hypertensive Diseases, (Y. Yamori, W. Lovenberg, and E. Fries, eds.). Raven Press, N.Y., 1979, pp. 497-504.

2. Yamori, Y., Nara, Y., Horie, R., Ooshima, A. and Lovenberg, W.: Pathophysiological role of taurine in blood pressure regulation in stroke-prone spontaneously hypertensive rats (SHR). In: The Action of Taurine on Excitable Tissue, (S. Schaffer and S. Baskin, eds.) Spectrum Press, N.Y., 1979 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01851-06 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of Tyrosine Hydroxylase in the Central Nervous System

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith Juskevich	NIH Postdoctorate Fellow	HE NHLBI
OTHER:	James P. O'Callaghan	Research Associate	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The primary goal of these studies is an understanding of the regulation of tyrosine hydroxylase in the central nervous system. We have previously shown an activation of the soluble enzyme by phosphorylation or catecholamine removal. Tyrosine hydroxylase activity is now being studied in a synaptosomal system, which provides conditions more similar to those found in vivo. Synaptosomes prepared from mouse whole brain, rat striatum and rat cortex have been used to study the regulation of tyrosine hydroxylase. Addition of calcium increases tyrosine hydroxylase activity in synaptosomes from mouse whole brain and rat cortex, but not rat striatum. In addition, we have found that tyrosine hydroxylase activity in whole brain and cortical synaptosomes is affected by extrasynaptosomal tyrosine concentration, and that addition of calcium changes the kinetics of tyrosine hydroxylase for tyrosine. Tyrosine hydroxylase activity in striatal synaptosomes did not show this tyrosine dependency.

Objectives: As the rate-limiting enzyme in catecholamine biosynthesis in the central nervous system, tyrosine hydroxylase is intimately involved in the regulation of neurons utilizing catecholamine neurotransmitters. In previous reports we have described some aspects of regulation of tyrosine hydroxylase activity in soluble enzyme preparations. The objective of our continued studies is to better describe the mechanisms involved in regulation of tyrosine hydroxylase activity under conditions more similar to those found in vivo. This preparation allows us to study several of the factors involved in regulation of the enzyme, including concentration of substrate and cofactor, end-product inhibition, ion effects, and their interactions. Since many drugs which have been shown to affect norepinephrine and dopamine turnover in vivo have not been shown to affect tyrosine hydroxylase activity using a soluble enzyme assay, we will investigate the effects of drugs administered in vivo on tyrosine hydroxylase activity in synaptosomal preparations.

Methods: Synaptosomes were prepared from mouse whole brain and rat striatum using standard procedures. The crude synaptosomal pellet or synaptosomes purified on a sucrose density gradient were resuspended in a modified Krebs buffer without calcium and containing 0.32 M sucrose. Tyrosine hydroxylase activity was quantitiated by measuring formation of tritiated water after addition of ^{3}H-tyrosine. For the standard assay each tube contained 150-250 μg protein, 5 x 10^{-5} M unlabeled tyrosine and Ca^{2+}-free or Ca^{2+}-buffer (final Ca^{2+} concentration = 1 mM). Tyrosine concentrations were varied from 5 x 10^{-6} M to 1 x 10^{-4} M. Tubes were incubated at 37°C for 10 min. The reaction was stopped with 0.4 ml 5% TCA. Samples were put over columns containing Dowex 50 x 4, activated charcoal and Dowex 1 x 2; the columns were washed twice with 0.7 ml H_2O. The effluent was collected in scintillation vials and ^3H_2O was quantitated by liquid scintillation spectrometry.

Uptake of tyrosine and Ca^{2+} into synaptosomes was determined by putting the reaction mixture over a Millipore filter. The filters were then dissolved in scintillation fluid and the amount of ^{3}H-tyrosine or $^{45}Ca^{2+}$ taken up was determined by liquid scintillation spectrometry.

The effects of Ca^{2+} on basal catecholamine release was determined using synaptosomes preloaded with ^{3}H-dopamine (striatal) or ^{3}H-norepinephrine (whole brain and cortical).

Major Findings: Tyrosine uptake into the synaptosomal preparations was rapid and essentially complete after two minutes exposure to various tyrosine concentrations (5 x 10^{-6}, 1 x 10^{-5}, 2 x 10^{-5}, 5 x 10^{-5} and 1 x 10^{-4}M). Tyrosine uptake was essentially linear over this concentration range.

In synaptosomes obtained from mouse whole brain and rat cortex the rate of tyrosine hydroxylation, expressed as pmole DOPA/mg protein/min, is dependent on the concentration of tyrosine in the external media. The rate of formation of DOPA is linear up to 50 μM and begins to be saturated at 100 μM. Conversely, in synaptosomes obtained from rat striatum the rate of tyrosine hydroxylation is not dependent on the concentration of external tyrosine, the relationship between tyrosine hydroxylation and tyrosine concentration being flat over the range of tyrosine concentrations tested.

The rate of tyrosine hydroxylation can also be affected by addition of Ca^{2+} into the incubation medium. In mouse whole brain and rat cortical synaptosomes addition of Ca^{2+} increases tyrosine hydroxylase activity. The rate of tyrosine hydroxylation was higher at each concentration of tyrosine except for 100 μM in mouse whole brain and 5 and 10 μM in rat cortex. The rate of tyrosine hydroxylation in striatal synaptosomes was unaffected by addition of Ca^{2+} to the incubation medium. This difference is not due to altered Ca^{2+} uptake since the uptake of ^{45}Ca into mouse whole brain and rat striatal synaptosomes was not significantly different.

To further study the discrepancy between the effect of tyrosine and Ca^{2+} on tyrosine hydroxylase activity in these synaptosomal preparations, we studied tyrosine uptake and basal catecholamine release.

Tyrosine uptake is essentially linear and similar in both rat striatal and mouse whole brain synaptosomes. In neither preparation did addition of Ca^{2+} cause an increase in tyrosine uptake. In addition it was found that catecholamine release is the same in the presence and absence of Ca^{2+} in all three synaptosomal preparations.

The exact mechanism of the Ca^{2+}-activation of tyrosine hydroxylase is therefore still unclear, however, it is possible that addition of Ca^{2+} results in activation of Ca^{2+}-activated kinases, resulting in increased phosphorylation of the enzyme.

Significance to Biomedical Research and Institute Program: The regulation of tyrosine hydroxylase activity has been studied in soluble enzyme preparations in an attempt to understand the regulation of this enzyme in vivo. Several important control mechanisms have been defined, such as phosphorylation, cofactor concentration and catecholamine end-product inhibition. However, the soluble enzyme preparation seems to lack some sensitivity in that the enzyme is removed from conditions normally found in vivo, as evidence by the difficulty in observing effects of drugs, known to effect catecholamine turnover or synthesis, on tyrosine hydroxylase activity. The synaptosomal preparation used in these studies provides us with a means of studying the regulation of tyrosine hydroxylase under conditions similar to those found in vivo. Such a system can provide important information concerning the interaction of drugs with central catecholaminergic neural systems.

The regulation of tyrosine hydroxylase may be important in disease states with central nervous system involvement. For example, increasing brain tyrosine concentration has been shown to lower blood pressure in spontaneously hypertensive rats. The work reported here may provide an explanation for this phenomenon.

Proposed Course of Project: Since differences were found between whole brain and striatum, the regulation of tyrosine hydroxylase activity in synaptosomal preparations from hypothalamus and pons medulla, as well as striatum and cortex will be studied. Further characterization will include further study of tyrosine and ion interactions with tyrosine hydroxylase activity as well as cofactor concentrations, end-product inhibition and phosphorylation of synaptosomal proteins.

Publications:

1. Young, R.A., Robinson, D.S., Vagenakis, A.G., Saavedra, J.M., Lovenberg, W., Krupp, P.P. and Danforth, E., Jr.: Brain TRH, monoamines, tyrosine hydroxylase, and tryptophan hydroxylase in the woodchuck, *Marmota Monax*, during the hiberation season. *Comparative Biochem. and Physiol.* 63: 319-323, 1979.

2. Robinson, D.S., Campbell, I.C., Walker, M., Lovenberg, W., Statham, N.J., and Murphy, D.L.: Effects of chronic monoamine oxidase inhibitor treatment on biogenic amine metabolism in rat brain. *Neuropharmacology* 18: 771-776, 1979.

3. Raese, J.D., Edelman, A.M., Makk, G., Bruckwick, E.A., Lovenberg, W., and Barchase, J.D.: Brain striatal tyrosine hydroxylase: Activation of the enzyme by cyclic AMP-independent phosphorylation. *Comm. Psychopharmacol.* 3: 295-301, 1979.

4. Lovenberg, W., Kuhn, D.M., Juskevich, J.: Neuronal systems and their impact on blood pressure regulation. *In*: *Proceedings of the Innisbrook Conference* - Fundamental Fault in Hypertension, Elsevier, (In press)..

5. Juskevich, J., and Lovenberg, W.: Neuronal regulation of blood pressure. *In*: *Biochemical Actions of Hormones*, Vol. 8, Academic Press, Inc. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01859-09 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Characterization and Mechanism of Action of Dopamine-β-Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sikta Pradhan	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Dopamine-β-hydroxylase (DBH) from bovine adrenal is purified to an apparent homogeneous form by passing through a column of DEAE cellulose, concanavalin A sepharose and sephacryl S-300 successively. On gel electrophoresis, this preparation of DBH shows only one band in the presence of 10% SDS, but two distinct bands in the absence of SDS. This result suggests that probably there is a contaminating protein with DBH having a similar molecular weight but with different charges as that of DBH. There is no significant decrease in enzyme activity of DBH after treatment with glycosidases to remove carbohydrate moiety from the enzyme molecule. The specific activity of this preparation of DBH is 22 μmoles/min/mg protein.

PHS-6040
(Rev. 10-76)

Objectives: The main objective of this project is to purify DBH in pure form for the determination of the importance of carbohydrate components on its biological activity.

Methods: Dopamine-β-hydroxylase (DBH) is isolated from bovine adrenal medulla according to Foldes et al., (Biochem. J. 126: 1209-1219, 1972). It is purified by passing through a column of DEAE cellulose, concanavalin A sepharose and Sephacryl S-300 successively. The major peak fraction containing DBH activity is concentrated by an Amicon diaflo ultrafilter, XM100A which generally retains proteins with molecular weights higher than 100,000 dalton. The homogeneity of the purified preparation is examined by gel electrophoresis both in the absence and presence of SDS (10%). This apparently purified DBH is treated with glycosidase enzymes prepared from Diplococcus pneumoniae, Type 1, to cleave the carbohydrate moiety from DBH molecule.

Major Findings: Our preliminary results show that the carbohydrate moiety can be cleaved without any loss of biological activity of DBH. No work has been done on this project in the current year.

Significance to Biomedical Research and Institute Programs: DBH is the final enzyme in the catalytic pathway for the synthesis of norepinephrine. This enzyme is located in the synaptic and chromaffin granule vesicles. Upon nerve stimulation it is released with the neurotransmitter by an exocytotic process. Hyperactivity of the sympathetic nervous system has been implicated in the development and maintenance of essential hypertension in man and experimental models of hypertension in animals. A clear knowledge of the molecular structure of DBH is required for a complete understanding of its role in both the normal and pathologic state.

Proposed Course of Project: There are evidences that the amount of serum DBH is different in different individuals. This fact may be related to the current hypothesis that sialic is essential for continued viability of the glycoprotein in the circulation. On treating the native protein with neuraminidase, galactose is exposed as the terminal nonreducing sugar of the protein-linked carbohydrate chains and serves as a specific determinant for the hepatic recognition of the sialic acid-deficient molecules. Hence, our objectives will be to determine the serum survival rate of DBH both native and treated with neuraminidase or glycosidases.

Publications:

1. Major, L.F., Lerner, P., Goodwin, F.K., Ballenger, J.C., Brown, G.L. and Lovenberg, W.: Dopamine-β-hydroxylase in cerebrospinal fluid: Relationship to personality measures. Arch. Gen. Psychiat. 37: 308-310, 1980.

2. Rosenberg, R.C., and Lovenberg, W.: Dopamine-β-Hydroxylase: Essays in Neurochemistry and Neuropharmacology, Vol. 4., M.B.H. Youdim, W. Lovenberg, D.F. Sharman and J.R. Lagnado, (eds.), 1980, John Wiley and Sons, Ltd., pp. 163-209.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01865-05 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Calcium-Calmodulin Dependent Activation of Tryptophan Hydroxylase by ATP and Magnesium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	James O'Callaghan	Staff Fellow, PRAT Program	HE NHLBI
	Judith Juskevich	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tryptophan hydroxylase [EC 1.14.16.4; L-tryptophan, tetrahydropteridine: oxygen oxidoreductase (5-hydroxylating)] in rat brainstem extracts is activated 2 to 2.5-fold by ATP and Mg^{++} in the presence of subsaturating concentrations of the cofactor, 6-methyltetrahydropterin (6MPH$_4$). The activation requires Mg^{++} and ATP but is not dependent on either cAMP or cGMP. The effect of ATP and Mg^{++} on enzyme activity was enhanced by μM concentrations of Ca^{++} and totally blocked by EGTA. Finally, removal of calmodulin from the brain extracts by affinity chromatography on a column of fluphenazine-Sepharose rendered tryptophan hydroxylase unresponsive to activation by ATP-Mg^{2+}. The re-addition of calmodulin restored the ATP-Mg^{2+}-induced activation only in the presence of Ca^{2+}. Drugs which bind calmodulin in vitro also block the ATP-Mg^{2+} effect on tryptophan hydroxylase.

Objectives: It has been demonstrated that both tyrosine hydroxylase (Lovenberg et al, PNAS 72: 2955-2958, 1975) and tryptophan hydroxylase (Kuhn et al, BBRC 82, 759-766, 1978) can be activated under in vitro conditions which favor protein phosphorylation. The activation of tryptophan hydroxylase is somewhat unique though, requiring Ca^{2+} and demonstrating no apparent dependence on cyclic nucleotides (Kuhn et al, 1978). The purpose of these continuing studies was to determine if calmodulin, the heat-stable calcium binding protein, played a role in the activation of tryptophan hydroxylase by phosphorylating conditions.

Methods: Male Sprague-Dawley rats were decapitated and the mesencephalic tegmentum, which contains essentially all of the serotonin [5-HT] containing perikarya in this species, was rapidly dissected from the brain, frozen on solid CO_2 and stored in liquid N_2. Tryptophan hydroxylase was assayed by the method of Friedman et al (J. Biol. Chem. 247: 4165, 1972) as modified by Baumgarten et al (J. Neurochem. 21: 251, 1973). For phosphorylating conditions, the following substances were added to the incubation mixture (in the final concentrations indicated): ATP (0.5 mM) and Mg^{++} acetate (5 mM).

The fluphenazine-Sepharose affinity matrix was prepared and used according to the method of Charbonneau and Cormier (BBRC 90: 1039-1047, 1979). SDS-polyacrylamide slab gel electrophoresis was carried out according to the method of O'Farrell (JBC 250: 4007-4021, 1975).

Major Findings: Exposure of tryptophan hydroxylase to phosphorylating components leads to a 2 to 2.5-fold stimulation of catalytic activity. This effect if rapidly reversible. ATP, cAMP, and Mg^{++} reduce the apparent K_m for $6MPH_4$ from 0.21 mM to 0.09 mM while having no effect on V_{max}. Kinetics for the substrate tryptophan were not changed. The stimulation of enzyme activity was dependent on ATP and Mg^{++}, but not upon cAMP.

The divalent cation calcium was also found to play an important role in the activation of tryptophan hydroxylase. Concentrations of Ca^{++} as low as 5-10 μM stimulate the ATP-Mg^{++} effect. Homogenization of brain tissue in the presence of EGTA prevented the activation of tryptophan hydroxylase by ATP-Mg^{++} which, in turn, can be reinstated by the addition of Ca^{++} in excess of EGTA. The addition of EGTA to the reaction mixtures also blocks the ATP-Mg^{++} activation. Chromatography of enzyme extracts on a column of fluphenazine-Sepharose, a process which removes endogenous calmodulin, rendered the hydroxylase unresponsive to ATP-Mg^{2+}. The external addition of calmodulin (1 μM) to chromatographed (calmodulin-free) enzyme restored the responsiveness of the enzyme to ATP-Mg^{2+}. Restoration by calmodulin was totally dependent on calcium. The addition to the phosphorylation reaction mixture of drugs which are known to bind to calmodulin also block the activation of the hydroxylase by ATP-Mg^{2+}. The drugs most effective in this regard come exclusively from the family of agents used as antipsychotics. The drugs used and the order of their inhibitory potencies (IC_{50}) are as follows: pimozide>fluphenazine>trifluoperazine>cis-flupenthixol>haloperidol>penfluridol>chlorpromazine. The inclusion of calmodulin prevented the inhibitory effects of the antipsychotic drugs on the activation of tryptophan hydroxylase by ATP-Mg^{2+}.

Significance to Biomedical Research and Institute Programs: Phosphorylation of a protein (tryptophan hydroxylase) may play a primary role in converting tryptophan hydroxylase to the low K_m, activated form. This mechanism is potentially very important in the *in vivo* regulation of TPH activity and correspondingly, in 5-HT synthesis. Since TPH is not inhibited by its end-product serotonin, it is not likely that changes in the intra-neuronal concentrations of serotonin would alter enzyme activity except under very extreme circumstances. Therefore, the activation of TPH by a protein phosphorylating system is perhaps the single most important mechanism by which TPH responds to increased demands for 5-HT.

The activation of tryptophan hydroxylase by ATP-Mg^{++} certainly suggests that a phosphorylation reaction is involved. This mechanism is novel, however, in that it is not dependent on cyclic nucleotides. Furthermore, Ca^{++} and calmodulin were found to play an important role in the activation. The present use of ligand specific affinity chromatography to identify a calmodulin dependent enzyme reaction represents the first such use of this methodology. Finally, the ability of antipsychotic drugs to inhibit the *in vitro* activation of tryptophan hydroxylase suggests that the *in vivo* mechanism of action of this class of drugs may include binding to calmodulin.

Proposed Course of Project: The following experiments are planned to more completely assess the role of calmodulin in tryptophan hydroxylation.

1. Purified calmodulin will be covalently bound to Sepharose-4B and this affinity matrix will be used to purify any protein kinases binding to the gel.

2. Rats will be treated chronically with a long acting antipsychotic, fluphenazine decanoate. At weekly intervals, groups of treated subjects will be sacrificed and the brains will be assayed in the presence and absence of phosphorylating components.

Publications:

1. Kuhn, D.M., O'Callaghan, J.P., Juskevich, J., and Lovenberg, W.: Activation of brain tryptophan hydroxylase by ATP-Mg^{2+}: Dependence on calmodulin. PNAS, 1980, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01867-05 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Kinetic Regulation of Rat Striatal Tyrosine Hydroxylase (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sikta Pradhan	Staff Fellow	HE NHLBI
OTHER:	Larry Alphs	Research Assistant	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.3	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been observed that pH of the reaction medium has an important influence on the kinetic properties of tyrosine hydroxylase. This enzyme appears to exert in at least two activity states. The highly active form (phosphorylated) has a low K_m for the hydroxylase cofactor and pH optimum of 6.2, whereas the inactive form has a pH optimum of 5.8 and a poor affinity for the cofactor. We have observed that dopamine receptor blockade in vivo results in a rapid activation of tyrosine hydroxylase in the dopaminergic terminals in the striatum. Because of the differential pH optima, the maximum regulatory effect are seen at pH values higher than 6.2. This study has revealed important mechanisms underlying the mechanism of a number of antipsychotic drugs.

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PHS-6040
(Rev. 10-76)

Objectives: The objective of this project is to study the kinetic regulation of tyrosine hydroxylase in the rat striatum. To do this we are seeking to determine the optimum conditions under which the drug-induced changes in tyrosine hydroxylase kinetics are observed. An attempt is also made to determine the time at which an apparent tolerance to the effects of haloperidol develops.

Method: Tyrosine hydroxylase is assayed using a slight modification of the tritium release method which has been described previously by Lovenberg, et al. In brief, activity is measured by determining the amount of tritiated water formed during the conversion of tritiated tyrosine to DOPA. In our assay a freshly prepared potassium phosphate-ascorbate buffer is used. This buffer is prepared by mixing 0.5 M $K_2H\ PO_4$ with 0.4 M ascorbic acid (2:1, v/v) and adjusting the pH to a value such that, after the addition of the remaining components of the assay system, the desired final pH of the reaction mixture is obtained.

The tissue extracted is purified through a Dowex (50 x 4, hydrogen form) column (0.6 X 2.0 cm) equilibrated with homogenizing buffer to remove endogenous catecholamines before the estimation of tyrosine hydroxylase activity.

Major Findings: Kinetic properties of tyrosine hydroxylase have been demonstrated to be markedly affected by pH of the reaction medium. As the pH of the reaction mixture is increased a marked increase in K_m is observed at higher pH (6.0-6.6) than the optimum pH for unactivated tyrosine hydroxylase (5.8-6.0).

When rats are treated subacutely for 10 days with haloperidol (1.0 mg/kg) and the kinetic parameters of tyrosine hydroxylase activity are examined at 1 hour and 23 hours after the last injection, the K_m is decreased from control values at 1 hour and rises above the control value at 23 hours. In acutely treated animals there is a decrease from control in K_m at 1 hour after injection which returned to control levels by 23 hours.

In order to determine if a specific subgroup of dopamine receptors is responsible for these changes, we repeated the acute and subacute experiments described above using fluphenazine (a more specific antagonist of beta-DA receptors) and sulpiride (a selective antagonist of alpha-DA receptors). The effects of fluphenazine in changing the rats striatal kinetics are in the same direction as those produced by haloperidol. But sulpiride decreases in striatal tyrosine hydroxylase activity (with an increase in K_m) in case of acute treatment and no significant change in tyrosine hydroxylase kinetics is observed in case of subacute treatment (the tyrosine hydroxylase activity was measured after 23 hours after the last injection).

To determine the approximate time needed to develop an apparent tolerance to the effects of haloperidol, rats received subacute treatment followed by a drug-free interval of 1,9 or 15 days. A reduced value in K_m is observed 1 hour after challenge with an eleventh injection when the drug-free interval is short. There is no change in K_m when the drug-free interval extends to 2 weeks. In similarly treated animals, which do not receive an acute challenge

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with haloperidol, the K_m of TH for $6MPH_4$ is elevated 23 hours after the last of 10 injections and continues to rise when the drug-free interval extends to 1 week. Thereafter, the K_m decreases towards that seen in control, untreated animals.

These results suggests that an apparent tolerance to the effects of haloperidol develops 2 weeks following the cessation of 10 days of treatment with haloperidol.

Significance to Biomedical Research and Institute Program: Tyrosine hydroxylase is the rate-limiting enzyme in the catecholamine biosynthesis. Hence, considerable attention has been focused on the mechanism of kinetic regulation of this enzyme. The studies of the mechanisms by which it is regulated are potentially of great importance to the understanding of both normal and pathologic states of the central nervous system. Among the pathologic states that might potentially be affected by these studies are conditions so diverse as essential hypertension, Parkinson's disease, Huntington's chorea and schizophrenia.

Proposed Course of Project: Since current literatures indicate that other enzymes, such as cAMP-dependent kinase, adenylate cyclase and calmodulin are involved in the regulation of tyrosine hydroxylase activity, future work should be directed towards the observation of the effects of different drugs on these enzymes and correlate them with the regulation of tyrosine hydroxylase kinetics obtained under similar conditions.

Publications:

1. Lovenberg, W., Alphs, L., Bose-Pradhan, S., Bruckwick, E. and Levine, R.: Long-term haloperidol and factors affecting the activity of striatal tyrosine hydroxylase. In: Advances in Psychopharmacology, Raven Press, N.Y., 1979 (In press).

2. Pradhan, S., Alphs, L. and Lovenberg, W.: Characterization of haloperidol mediated effects on rat striatal tyrosine hydroxylase. Neuropharmacology, 1980 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01872-04 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pteridine Cofactor Levels in Biologic Fluids

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Levine	Pharmacologist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Donald Calne	Chief, Exp. Ther. Branch	ETB NINCDS
	Leonard Miller	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)
NIMH, Biological Psychiatry Branch; NINCDS, Experimental Therapeutics Branch

LAB/BRANCH
Hypertension-Endocrine Branch

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	0.5	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The biosynthesis of catecholamines is dependent upon the hydroxylase cofactor tetrahydrobiopterin (BH_4). Since the concentration of BH_4 in brain tissue is thought to be less than saturating for tyrosine hydroxylase and that the level of this compound in CSF may be reflective of the concentration in brain, it was of value to measure the BH_4 content in CSF of selected subjects.

This study was undertaken to detect and monitor CSF hydroxylase cofactor activity using a phenylalanine hydroxylase assay. Our studies indicate hydroxylase cofactor activity declines with age in both normal and Parkinsonian patients. No significant alteration was found in CSF cofactor levels of schizophrenic or affective disorder patients. We have reported that patients with certain neurological diseases, most notably Parkinson's disease have reduced levels of cofactor in their CSF. In addition, certain patients with generalized dystonia have relatively low CSF cofactor content.

PHS-6040
(Rev. 10-76)

Objectives: Since a variety of diseases may result from defects in monoamine synthesis, it is of interest to assess one possible index of in vivo aminergic function, i.e., CSF hydroxylase cofactor activity. Studies of neurologic and psychiatric patients are in progress in collaboration with the Biological Psychiatry Branch, NIMH and the Neurology Service. In addition, we will be investigating cofactor levels in human CSF using an independent method of assay, a high-pressure liquid chromatographic system. Association between CSF cofactor activity and disease states, drug treatments and biologic variables such as age, sex etc. will be investigated. Our neurological studies on CSF cofactor have recently been expanded to include patients with dystonia, as there have been implications of abnormal aminergic function in this disease.

Methods: Two ml of CSF is freshly collected in an opaque polycarbonate tube, wrapped in aluminum foil to protect it from light and stored in liquid N_2 until time of assay. CSF hydroxylase cofactor content is measured by an existing radioenzymic assay which was modified in our laboratory as previously reported to achieve the necessary sensitivity.

CSF biopterin will be measured by a high pressure liquid chromatography system. Reduced and oxidized forms of biopterin can be separated and quantitated by this method. Iodine oxidation of biological fluids under acid conditions converts all the reduced forms of biopterin to the completely oxidized state. Iodine oxidation under basic conditions selectively oxidizes nonquinoid dihydrobiopterin to biopterin while converting the tetrahydro and quinoid dihydro forms of biopterin to other compounds which do not appear in the biopterin peak after HPLC separation. Total biopterin content is quantitated by peak height after separation on a reverse-phase C_{18} column.

Major Findings: We have been able to measure hydroxylase cofactor content by the enzymic assay in all CSF samples assayed from patients with various neurological diseases, schizophrenia, affective disorders, and normal control patients. Preliminary analysis of data collected from schizophrenic and affective disorder patients indicates no detectable alterations in CSF cofactor content when compared to normal controls. However, a larger sample population will be analyzed before these findings are determined conclusively. Results from the neurological and normal patients populations proved to be most interesting. We have previously reported that patients with Parkinson's disease have a reduced CSF cofactor content when compared to control patients and that CSF cofactor is positively correlated with CSF homovanillic acid content. Extending our studies to other neurological diseases, we observed a decreased CSF cofactor in Shy-Drager syndrome, Steele-Richardson syndrome, Huntington's disease, and pre-senile dementia. This decreased CSF cofactor in a variety of neurological disease indicates that a low CSF cofactor value probably reflects generalized aminergic cell loss from an unidentified cause rather than a primary defect in cofactor metabolism. In this regard, we have also reported a decrease in CSF cofactor with age in both the normal population and patients with Parkinson's disease. This finding is consistent with other reports of diminishing central catecholaminergic function with age and supports our contention that CSF cofactor levels could serve as an index of central aminergic cell loss over time.

Certain patients with generalized dystonia of the dominant inheritance pattern also exhibited relatively low CSF cofactor content. This finding along with our findings in Parkinson's disease has generated a great deal of interest in the possibility of pharmacological elevation of brain cofactor levels in certain patients with dystonia and Parkinson's disease.

Significance to Biomedical Research and Institute Programs: (1) It is important to identify the major determinants of monoamine synthesis and turnover in man, and to assess the relationships of these variables to biologic variables and disease states. (2) Measurement of CSF cofactor activities in patients has revealed cofactor deficiencies in certain neurological disease states where impaired monoamine metabolism has been shown to be an etiologic factor (such as basal ganglia degeneration). (3) This project will provide additional insight into mechanisms which control CNS monoamine levels and their relationships to pathologic conditions. (4) Our reports of a decreased CSF cofactor content with age in both the normal and Parkinsonian populations has increased our knowledge of changes which occur within the central nervous system during the aging process. (5) Our findings of reduced cofactor levels in parkinsonian and dystonic patients may provide the basis for successful replacement therapy with exogenous hydroxylase cofactor.

Proposed Course of Project: Assay of patient CSF specimens will be continued in order to enlarge our series of patients with neurologic and psychiatric diseases. We will continue our investigations into the relationship between CSF cofactor content and the condition of dystonia. The high pressure liquid chromatographic assay for biopterin will be set up in our laboratory to allow for a more specific assay for CSF biopterin content (see Methods). We are also investigating the possibility of having large quantities of biopterin synthesized for a clinical trial with selected neurological patients. The associated animal experiments which would be necessary as a prelude to such a clinical trial are also being examined. This project will be expanded to study mechanisms of endogenous cofactor synthesis which may lead to alternative methods of manipulating the disposition of cofactor within the brain.

Publications:

1. Van Kammen, D.P., Levine, R.A., Sternberg, D., Ballenger, J., Marder, S., Post, R. and Bunney, Jr., W.: Preliminary evaluation of hydroxylase cofactor in human spinal fluid: Potential biochemical and clinical relevance in the study of psychiatric disease. Psychopharm. Bull. 14: 51-52, 1978.

2. Levine, R.A., Williams, A.C., Robinson, D.S., Calne, D.B. and Lovenberg, W.: Analysis of hydroxylase cofactor activity in human cerebrospinal fluid of patients with Parkinson's Disease. In: Advances in Neurology, Raven Press, N.Y., 24: 303-307, 1979.

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3. Williams, A.C., Eldridge, R., Levine, R.A., Lovenberg, W., and Paulson, G.: Low CSF hydroxylase cofactor (tetrahydrobiopterin) levels in inherited dystonia. Lancet 2 9139: 410-411, 1979.

4. Eisler, T., Calne, D.B., Ebert, M.H., Kopin, I.J., Ziegler, M.G., and Levine, R.: Biochemical measurements during (-) deprenyl treatment of Parkinsonism. In: MAO Strucutre, Function and Altered Function, Murphy, D., Singer, T., and Vonkoff, R. (eds.), New York, Academic Press, pp 497-506, 1979.

5. Levine, R.A., Kuhn, D.M., Williams, A.C., and Lovenberg, W.: The influence of ageing on biogenic amine synthesis: The role of the hydroxylase cofactor. In: Influence of Age on the Pharmacology of Psychoactive Drugs, Raskin, A., Robinson, D.S., Levine, J. (eds.), Elsevier, North Holland (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01874-04 HE

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Adrenergic Neurons in the Brain (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	NHLBI HE
OTHER:	Willa B. Phyall	Research Associate	NHLBI HE
	M. Fujiwara	Visiting Fellow	NHLBI HE

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	0.8	0.2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The study of the nature and the role of epinephrine containing neuron in brain has been continued. A previously reported finding that phenylethanolamine N-Methyl transferase (PNMT) was higher than normal in spontaneously hypertensive rats was confirmed. Immunohistochemical analysis indicated that this increased activity was associated with an increase number of neurons rather than an increased activity per cell. We have also found that the brain PNMT activity can be enhanced by dexamethasone or a stressful situation. This increase in activity occurs over a short time course and suggest activation of preformed enzyme molecules. In related experiments we observed the effect of unilateral carotid artery ligation. Of interest was the relatively rapid increase (40%) in PNMT activity on the contralateral side of the brain.

PHS-6040
(Rev. 10-76)

Objectives: The cell bodies of the epinephrine containing neurons lie in clusters in the lower brainstem. The dorsal group C_2 are very near the area postrema whereas the C_1 group lies relatively close to the ventral surface. These groups are strategically located for a role in blood pressure regulation, although no such role has been identified to date. Since it had been reported that the activity of PNMT the marker enzyme for these cells, was higher in SHR than in normal rats we planned to determine whether this was accounted for by an increased number of cells. We also planned to investigate whether changes in the pattern of blood flow in the CNS by carotid artery ligation has an effect on PNMT levels.

Methods: For immunocytochemical analysis age matched stroke-prone SHR and Wistar Kyoto rats were perfused with a formaldehyde solution in buffered aulane containing a 0.05% glutasuldehyde. Starting at the Obex 50μ sections of the frausstein were prepared. The slices were stained with rabbit antisera and counterstained with FITC labelled anti rabbit gamma globulin. The number of fluorescent positive cells on alternate slices were counted independently by 3 individual who were not aware of the origin of the slices. In others experiments PNMT activity was measured by standard previously described techniques.

Major Findings: The previously described increase in PNMT in SHR was confirmed and extended to show that the stroke-prone SHR has an approximately 25% increase in the enzyme activity. In each of the pairs of SHR-SP and WKY examined the SHR-SP had significantly more PNMT positive cells. With 6 animals in each group WKY had mean value of about 700 cells whereas SHR-SP had about 900 cells. While there are a number of potential artifacts it seems clear that there are significantly more PNMT positive cells in the brainstem of the hypertensive rats. The relationship of the increased cell number to the development of hypertension is not known.

The mechanism by which the activity of PNMT is regulated and the relation to the activity of PNMT neurons remain to be determined. Recent studies have shown that acute stress can cause a rapid increase in PNMT activity in brain. Since Carotid artery ligation significantly alters cerebral hemodynamics and blood pressure it was of interest to examine its effect on brainstem PNMT. The enzyme activity was measured in the C_1 and C_2 regions on each half of the brainstem 6 hours after unilateral carotid artery ligation. This was done in both SHR and control WKy animals. Of interest was the observation that the enzyme activity on the contralateral side was increased by about 40% whereas only slight increases were seen on the unilateral side. The mechanism of this selective increase remains to be determined. It may simply represent a response to stress, or it could be related to the blood pressure changes that take place following ligation.

Significance to Biomedical Research and Institute Programs: It has been proposed that epinephrine neurons in the brain may be associated with mechanisms regulating blood pressure. The goal of this project is to understand how this neuronal system is regulated whether it does indeed have an impact on blood pressure.

Proposed Course: From the above studies it is clear that PNMT activity can be increased in a short period of time. Since it is unlikely to be due to new enzyme synthesis we plan to investigate potential mechanisms for activating this enzyme.

Publications:

1. Howe, P.R.C., Lovenberg, W., and Chalmers, J.P.: Increased number of PNMT-immunofluorescent nerve cell bodies in the medulla oblongata of stroke-prone hypertensive rats. Brain Res. (In press).

2. Phyall, Willa and Lovenberg, W.: The effects of hypophysectomy on the kinetics and regulation of PNMT in the brain and adrenal glands of rats. In: Catecholamines: Basic and Clinical Frontiers, Usdin, E., Kopin, I. and Barchas, J. (eds.) Pergamon Press, N.Y.,1979, pp. 180-182 (Vol. 1).
3. Keeton, T.K., Krutzsch, H. and Lovenberg, W.: A specific radioimmunoassay for 3-methoxy-4-hydroxyphenylethylglycol (MOPEG). In: Catecholamines: Basic and Clinical Frontiers, Usdin, E., Kopin, I. and Barchas; J. (eds.), Pergamon Press, N.Y., 1979, pp. 871-873 (Vol. 1).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01877-03 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparison of Molecular Kinetic and Regulatory Properties of Brain and Mass Cell Tryptophan Hydroxylase (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Robert C. Rosenberg	Staff Fellow	HE NHLBI
	Mary Ann Meyer	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The molecular parameters of tryptophan hydroxylase from rat brainstem and murine mast cell, were determined by gel filtration and sucrose density gradient ultracentrifugation. The enzyme from rat brainstem has a calculated molecular weight of 220,000 daltons, a Stokes radius of 55.6Å, a frictional ratio of 1.28, and a sedimentation coefficient of 9.63S. The mast cell enzyme has a molecular weight of 144,000 daltons, a Stokes radius of 50.3Å, a frictional ratio of 1.35, and a sedimentation coefficient of 6.97S. Evidence for catalytically active subunits was not found. The regulatory and kinetic properties of the brainstem and mast cell hydroxylase were also compared. The brain enzyme can be activated by calcium, SDS, trypsin, phospholipids, protein phosphorylation, and by heparin. Of these treatments only heparin activated the mast cell tryptophan hydroxylase. It appears that the tryptophan hydroxylase species from rat brainstem and murine mast cell represent distinct molecular entities.

PHS-6040
(Rev. 10-76)

Objectives: Tryptophan hydroxylase is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin. Despite numerous studies on the mechansims by which the activity of this important enzyme is regulated, little is known about the biophysical properties of tryptophan hydroxylase. The objectives of these experiments are to determine some of the molecular, kinetic and regulatory parameters of tryptophan hydroxylase from rat brainstem and murine mast cell, two tissues rich in this enzyme activity.

Methods: Male Sprague-Dawley rats were sacrificed by decapitation and brainstems were rapidly dissected from the brains and frozen on dry ice, and stored in liquid N_2 until assay. Mast cells were obtained from the ascitic fluid of BALB/cX DBA mice as described by Lovenberg et al. (Science 115:217-220, 1967). Cells were harvested, washed and frozen in N_2 until used for assay. Tryptophan hydroxylase was assayed by the methods reported by Baumgarten et al. (J. Neurochem. 21:251, 1973). Gel filtration (ultragel AcA34) and sedimentation experiments were performed essentially by the methods reported in Rosenberg and Lovenberg (Mol. Pharmacol. 13:652, 1977).

Major Findings: The major findings remain the same as described in last years report. It was reported from another laboratory that proteolytically treated tryptophan hydroxylase has catalytic properties which are quite similar to those of the mast cell hydroxylase described above. These data suggest that the mast cell enzyme may be a proteolytic product of the "parent" hydroxylase.

Significance to Biomedical Research and Institute Programs: These results represent the first systematic determination of the molecular parameters of tryptophan hydroxylase from rat midbrain and murine mast cell. The calculated molecular weight at least of the brain enzyme agrees quite well with the molecular weight of a highly purified tryptophan hydroxylase from rabbit brainstems and, as such, points out the usefulness of these procedures in studying the biophysical properties of a labile enzyme-like tryptophan hydroxylase even in a crude, high speed tissue supernatant fraction. These results also suggest that catalytically active subunits for at least the brain and mast cell enzyme do not exist under the present conditions. Taken together, these data indicate that the tryptophan hydroxylase enzymes from rat midbrain and murine mast cell represent distinct molecular entities.

The murine mast cell is a rich source of tryptophan hydroxylase and serotonin. However, very little is known about the mechanisms by which the activity of the mast cell enzyme is regulated (if it is regulated at all). These experiments have shown that the mast cell hydroxylase does not behave like the brain hydroxylase. Additional studies on the molecular mechanisms regulating tryptophan hydroxylase in the mastocytoma cells will help elucidate the means by which this malignant cell line expresses its efficient catalytic activity. These studies also lend valuable information on alternative mechanisms of control of the brain enzyme (e.g., end-product, inhibition, allosteric activation).

Proposed Course of Project: Using these same procedures, tryptophan hydroxylase from rat brainstem and murine mast cell will be partially purified so that the regulatory properties of each can be studied in more detail. For example, the effects of calcium, EGTA, thiols, and phosphorylating components on the kinetic properties and substrate specificity of each enzyme will be determined. The mast cell enzyme will also be studied more extensively to determine if the enzyme is synthesized as a smaller entity than the brain hydroxylase or if it is cleaved by a protease during the process of tumor infiltration of the cell.

Publications:

1. Kuhn, D.M., Rosenberg, R.C. and Lovenberg, W.: Determination of some molecular parameters of tryptophan hydroxylase from rat brainstem and murine mast cell. J. Neurochem. 33: 15-21, 1979.

2. Kuhn, D.M., Meyer, M.A., and Lovenberg, W.: Comparisons of tryptophan hydroxylase from a malignant murine mast cell tumor and rat mesencephalic tegmentum. Arch. Biochem. Biophys., 199: 355-361, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01879-03 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of the Ca^{2+}-Dependent Regulator (Calmodulin)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	H.-Y.T. Yang	Biochemist	LPP NIMH
	W. Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

Laboratory of Preclinical Pharmacology, NIMH, St. Elizabeth's Hospital, Washington, D.C. 20032

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Specific immunoglobulins directed toward calmodulin were produced in rabbits. An enzyme-linked immunosorbent assay was developed for the measurement of calmodulin. In rat striatum calmodulin occurs in membrane-bound and soluble form. In striatal supernatant fractions it exists in free and protein (presumably, PDE)-bound form. Hence the regulation of calmodulin was viewed in terms of biochemical processes which modulate the distribution of this protein. Chronic treatment with haloperidol increases the membrane-bound calmodulin content. Studies on the turnover-rate of this protein failed to show a change in its synthesis-rate. From these results it can be inferred that chronic treatment with haloperidol may modify the binding affinity of calmodulin to its binding site at the membrane.

Objectives: Calmodulin, a protein with high affinity binding sites for Ca^{2+} was first described by Cheung and subsequently purifed in several laboratories. Experimental evidence indicates that calmodulin regulates the activity of a number of enzymes including adenylate cyclase, phosphodiesterase, Ca^{2+}, Mg^{2+} ATPase and protein kinase. The enzyme activation requires an initial binding of Ca^{2+} to calmodulin and this complex subsequently binds to the enzyme. The structure, function and distribution of calmodulin indicates that in some neuronal systems it may serve as a regulatory link in coupling external stimuli to the cytoplasm in post-synaptic neuronal elements. There exist experimental evidence that the dopaminergic system in particular is associated with calmodulin. It has been shown that in striatum the dopamine-sensitive adenylate cyclase and cAMP-PDE are regulated by calmodulin. Furthermore, during supersensitivity the calmodulin content in synaptosomal membrane is increased. Since nothing is known on the regulation of calmodulin in the membrane-bound and soluble pool this project will be particularly directed toward studies on the regulation of calmodulin in both cellular compartments. Studies on the turnover-rate of calmodulin will help to reveal whether during supersensitivity of dopamine-receptors the accumulation of membrane-bound calmodulin is due to enhanced synthesis rate or to a blockade of release into the cytosol.

Methods: Preparation of antibody directed toward calmodulin. Calmodulin was purified from pig brain according to the procedure of Klee (Biochemistry 16: 1017-1024, 1977). For the production of immunoglobulins directed toward calmodulin, the purified protein was coupled to hemocyanin. This conjugate was emulsified in complete Freund's adjuvant and was injected intradermally into the back of rabbits. The injections were repeated at 2 week intervals.

Enzyme-linked immunosorbent assay (ELISA). Partially purified immunoglobulins (50% $[NH_4]_2SO_4$ saturation) were coupled with various concentrations of calmodulin or unknown calmodulin tissue extracts. The mixtures were transferred to microplates coated with calmodulin and incubated for a period of time. Horseradish peroxidase labelled anti-rabbit globulins were added to bind on the antigen-antibody complex. Thereafter, the microplates were incubated with a O-phenylenediamine-H_2O_2 reagent and the O.D. in each well of the microplates was measured with an ELISA spectrophotometer at 488 nm.

Studies on turnover-rate of calmodulin in striatum. After chronic treatment with haloperidol a cannula was implated into the lateral ventrical of rats. On the following day 0.50 mg cycloheximide was injected and 2 hours thereafter another 0.25 mg cycloheximide were given. At 6,8 and 12 hours thereafter the rats were killed and the calmodulin content was measured by micro-ELISA.

Major Findings: Highly specific immunoglobulins directed toward calmodulin showed little cross-reactivity with other species of Ca^{2+} binding proteins such as troponin C or Δ-light chain protein. Calmodulin occurs in high concentrations in the brain where it is particularly enriched in the synaptosomal membrane-fraction (∿ 14 μg/mg protein). In contrast, in adrenal medulla the calmodulin content is 0.40 μg/mg protein and in heart 0.10 μg/mg protein.

In the supernatant fraction from striatal homogenates two immunoreactive pools of calmodulin are present. Calmodulin elutes from Sephadex G-150 in two peaks. One peak elutes together with cAMP-PDE (molecular weight range of 158,000) whereas the second peak elutes together with low molecular weight proteins (25,000). The association of calmodulin with cAMP-PDE is Ca^{2+}-dependent since the PDE-calmodulin complex could be resolved by filtration on Sephadex G-150 equilibrated and eluted with 0.05 M Tris-buffer containing 1 mM EGTA. Chronic treatment with haloperidol increases the content of membrane-bound calmodulin. Measurements of turnover-rate of calmodulin show that the rate of decline of this protein is faster in saline treated rats than in haloperidol treated rats. Although the fractional rate constant was lower after haloperidol treatment, the turnover-rate was similar in both groups, because the steady-state concentration of calmodulin is higher in haloperidol treated rats. Thus, chronic treatment with haloperidol does not increase the synthesis-rate of calmodulin but may rather modify the binding affinity of calmodulin to its binding site at the membrane.

Significance to Biomedical Research and Institute Programs: The development of an enzyme-linked immunosorbent assay for calmodulin provides a new tool required to study the regulation of calmodulin content. Since the action mechanism of neuroleptic drugs, which act particularly on the extrapyramidal system, has been shown to be linked to cyclic=nucleotide metabolism studies on calmodulin-turnover rates will help to reveal some of the molecular features of their action mechanism.

Proposed Course of Project: This project will be terminated.

Publications:

1. Hanbauer, I. and Phyall, W.: Involvement of calmodulin in the modulation of dopamine receptor function. In: Long-term of Neuroleptics (Racagni, G., Spano. O., and Cattabeni, F., eds.) Raven Press, N.Y., Adv. Biochem. Psychopharmacol. 24: 133-138, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01881-03 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effects of Pharmacological Manipulations on the Disposition of Reduced Biopterin (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Robert Levine	Pharmacologist	HE NHLBI
OTHER:	Donald Kuhn	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.9	0.4	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The investigation of the major determinants of monoamine synthesis and turnover in vivo is of scientific interest because monoamine levels in the central nervous system (CNS) play critical roles in neuropsychiatric, neuroendocrine and cardiovascular diseases. Tyrosine and tryptophan hydroxylase are known to be the rate-limiting steps in the synthesis of catecholamines and serotonin, respectively. Current evidence suggests that the in vivo rate of synthesis of these compounds may be mediated by the concentration of the hydroxylase cofactor, tetrahydrobiopterin (BH_4). We have previously reported a significant positive correlation between hydroxylase cofactor content and total hydroxylase enzyme activity across rat brain areas. In addition, there is an unusually high content of cofactor in certain neuroendocrine tissues, which may indicate a potential neuroendocrine role for the cofactor. Using the catecholaminergic neurotoxin, 6-hydroxydopamine, we now report >85% reduction in reduced cofactor content in both the substantia nigra and striatum after intranigral injection. Kainic acid injected in the striatum, which destroys striatal cell bodies and interneurons, caused a modest decrease in striatal cofactor. These results indicate that reduced cofactor is highly concentrated within dopaminergic neurons of the nigrostriatal system.

PHS-6040
(Rev. 10-76)

Objectives: In order to examine the potential roles of brain BH_4, it is necessary to determine the levels of this compound in discrete brain areas. In addition, it is of interest to examine both the pharmacologic and physiolobic manipulations of BH_4 levels correlated with changes in hydroxylase enzyme activities. Manipulation of BH_4 levels may be more easily accomplished through a detailed understanding of its endogenous biosynthesis. The use of chemical neurotoxins will allow the study of the site of endogenous biopterin synthesis as well as the degree of colocalization of the cofactor with the hydroxylase enzymes in aminergic neurons.

Methods: Male Sprague-Dawley rats were decapitated and brains were quickly removed in the cold and dissected into the following areas: pons-medulla, striatum, hippocampus, tegmentum, tectum, cerebral cortex, cerebellum, thalamus, hypothalamus, pineal, and septum. In addition, the anterior and posterior pituitary, retina and adrenals were also dissected. Tissue was stored in liquid nitrogen until time of assay. Tissue samples were homogenized in 1.0 N HCl and aliquots of the supernatant fraction were lyophilized to dryness. Reaction mixture contains .013 units sheep liver quinoid dihydropterin reductase (QDPR), 0.85 μMol NADH, 36 units of highly purified phenylalanine hydroxylase, 10 μmol KPO_4 pH 6.8, 400 units catalase, and 4-^{3}H-L-phenylalanine (30 μC/μmol) in a final volume of 70 μl. Following incubation for 45 min. at 30°C, the reaction mixture is cooled at 0P in an ice bath, and the reaction arrested by addition of 0.1 ml of 1.2 M sodium acetate buffer, pH 5.5. To release any tritium in the 3-or 5-position of the tyrosine formed, 25 μl of N-iodosuccinamine (50 mg/ml in DMSO) is added to the cooled samples. After 5 min the released tritium (as ^{3}HOH) is collected by passage of the reaction mixture over a 0.6 x 3 cm Dowex 50-H + exchange resin. The column is washed twice with 0.70 ml of water, and the total eluent collected in a scintillation vial to which 15 ml of scintillation cocktail is added. A calibration curve using BH_4 standards in 1.0 N HCl is run concomitantly. A UV spectrum is obtained on a 50 μM solution of BH_4 in .01 N NCl just prior to the assay to quantify the concentration of reduced cofactor in the cofactor stock solution.

Neurosurgical procedures described below were performed on male Sprague-Dawley rats (200-300 g) using a Kopf sterotaxic instrument. A 10 μl Hamilton syringe was used to inject 6-hydroxydopamine (6OHDA-8 μg in 4 μl saline with 0.2 mg/ml ascorbic acid under argon atmosphere at 4°C and protected from light) into the left substantia nigra (coordinates from Bregma: AP=5.2 mm, ML= +2.0 mm, DV=-7.1 mm). Animals lesioned with 6OHDA were injected subcutaneously with apomorphine (1.0 mg/kg), a dopamine receptor agonist, one week following surgery; animals were sacrificed one week after injection of apomorphine. This drug induces contralateral rotational behavior which indicates a successful nigrostriatal lesion. Kainic acid (2 μg in 1 μl saline on ice and protected from light) was injected into the left striatum (coordinates from Bregma: AP=0.0 mm, ML=+3.0 mm, DV=-4.0 mm) of other rats. The decrease in substance P (SP) content was measured in the anterior striatum and nigra according to the method of Hanson and Lovenberg to confirm a successful striatonigral lesion by kainic acid. The brains of 6OHDA-lesioned rats were dissected into 1 mm thick slices with the aid of a plexiglass brain block and the striatum was dissected out of the appropriate slices.

Major Findings: We have reported significant correlations between BH_4 content and both tyrosine (r=0.64) and tryptophan (r=0.58) hydroxylase enzyme activities across various rat brain areas. The correlation between BH_4 content and total hydroxylase activity (tyrosine + tryptophan) was even better (r=0.80). Our determinations, however, revealed unusually high cofactor concentrations in the hypothalamus, pituitary, and pineal gland when compared to hydroxylase enzyme activity. These areas are known to be involved in many neuroendocrine functions. The high cofactor content in these areas indicates a potential alternative role for BH_4 other than serving as hydroxylase cofactor, possibly related to neuroendocrine function. The distribution of cofactor content within the individual lobes of the pituitary was also examined. The anterior pituitary had as much cofactor content (2.0 pmol/mg protein) as some of the low cofactor-containing brain areas and yet, as reported by others, there is minimal hydroxylase enzyme activity present in the anterior pituitary. The intermedio-posterior pituitary had much higher cofactor content (25 pmol/mg protein) which might be expected due to the innervation by aminergic neurons projecting from the hypothalamus. Our results demonstrate that this relationship of very high cofactor compared to hydroxylase enzyme activity is most marked in the anterior pituitary.

Destruction of nigrostriatal dopaminergic neurons by unilateral injection of 6-hydroxydopamine (6OHDA) into the left substantia nigra (SN) caused greater than 85% reduction in reduced cofactor content in both the SN and the striatum when compared to these same areas on the contralateral control side. Kainic acid injected into the left striatum of other rats, which destroys striatal cell bodies and interneurons while sparing nerve terminals, caused a modest decrease in striatal cofactor content and no change in the SN on the lesioned side. Our results imply that the cofactor is highly concentrated in dopaminergic neurons of the nigrostriatal system. These results also indicate the likelihood that the hydroxylase cofactor is synthesized predominantly by the dopamine cells within the nigrostriatal system.

Significance to Biomedical Research and Institute Programs: Understanding the levels of BH_4 in discrete brain areas may unveil previously undiscovered physiological roles of this compound.

It is known that patients with Parkinson's disease have significantly lower CSF BH_4 levels. An understanding of the endogenous biosynthesis of BH_4 may allow for alternative methods for manipulation of BH_4 levels in these patients, possibly improving their clinical symptoms.

Our studies will lead to a better understanding of what cell types in brain synthesize the cofactor as well as the nature of certain drug effects on the levels of the cofactor in selected brain areas and how these changes in cofactor levels relate to hydroxylase enzyme activity.

Proposed Course of Project: Further investigation is necessary into determining BH_4 levels in more discrete brain areas as well as other tissues which express high BH_4 content. More sophisticated micro dissecting techniques will be employed. A high pressure liquid chromatographic assay system has been recently set up in our laboratory for BH_4 measurements to assist us in our studies on BH_4.

In addition, techniques will be developed to study the biosynthetic pathway of BH_4 and the effects of pharmacological agents on the biosynthesis of BH_4 will be examined. Experiments designed to elevate brain reduced cofactor levels by injecting BH_4, 6-methyltetrahydropterin, and dimethyl-tetrahydropterine will be carried out.

The mechanism by which amphetamine and reserpine alter cofactor levels in the striatum will be examined using both the enzymic assay and HPLC.

Neurochemical lesion studies will be expanded using 5.7 dihydroxytryptamine to lesion serotonergic neurons to examine the degree of colocalization of cofactor and tryptophan hydroxylase.

Publications:

1. Levine, R.A., Kuhn, D.M. and Lovenberg, W.: Regional distribution of hydroxylase cofactor in rat brain. J. of Neurochem. 32: 1575-1578, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01884-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Enkephalin-like peptides and cardiovascular control

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ingeborg Hanbauer	Pharmacologist	HE NHLBI
OTHER:	G.D. Kelly		HE NHLBI
	H-Y.T. Yang	Biochemist	LPP NIMH
	T.D. Hexum	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)
Laboratory of Preclinical Pharmacology, NIMH, St. Elizabeth's Hospital
Washington, D.C.

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.4	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES X☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Enkephalin-like peptides immunoreactive to (met^5) or (leu^5)-enkephalin are contained in adrenal chromaffine cells and in splanchnic nerve plasma obtained from dogs with an indwelling catheter in the adrenal lumbar vein contains a higher content of enkephaline-like peptides than plasma from jugular or femoral vein. Electrical stimulation of the splanchnic nerve increase the enkephaline-like peptide content in adrenal venous plasma. Injection of morphine also increases the plasma content of these peptides. Pretreatment of dogs with naloxone or prior splanchnic nerve transection prevents this increase.

PHS-6040

Objectives: Recent reports suggest that met-enkephalin-like peptides may function as neurotransmitters or neuromodulators in sympathetic ganglia (DiGuilio, A.M. et al., Neuropharmacol. 17:989-992, 1978) and adrenal medulla (Yang, H-Y.T. et al., Fed. Proc.; Schultzberg, N. et al., Neurosci. 3:1169-1186, 1978). These met-enkephalin-like peptides, are concentrated in adrenal chromaffin granules, although the splanchmic nerve innervating the cortex and medulla of the adrenal gland also contains these peptides. Studies on primary cultures of bovine chromaffine cells showed that met-enkephalin-like peptides can be released by micotinic receptor stimulation (Kumabura et al., Nature 283:489-492, 1980. When released from afferent axons, they may act on opiate receptors located in peripheral nervous tissue.

The principal effect of narcotics upon the circulation seems to be hypotension. This is mainly caused by vasodilation and peripheral pooling of blood, although the mechanism involved is vaguely understood. The vasodilatory action of morphine, therefore, invites to speculate that opiate-like peptide hormones were to be secreted from sympathetic nerves or adrenal chromaffin granules, and might cause vasodilatation by acting on distant receptors.

In view of the possible role of met-enkephalin-like peptides in the regulation of sympathetic transmission and vascular tone, we have studied the content of met-enkephalin-like peptides in adrenal gland and in the adrenal venous outflow after electrical stimulation of the splenchnic nerve or direct stimulation of nicotinic or muscarinic receptors.

Methods: Collection of adrenal venous blood. Female American foxhounds (1 year old) were fitted with an indwelling catheter in the left lumbar adrenal vein. The sampling of adrenal venous blood was carried out both in conscious and anesthetized dogs. In some animals the spaldinic nerve was transected at least 3 days prior to the experiment.

Blood Pressure Measurements. Dogs were anesthetized with pento-barbitol and cannulae were implanted into the femoral vein and femoral artery. The mean arterial blood pressure was recorded from normal and reserpinized dogs before and during electrical stimulation of the splanchnic nerve.

Measurement of met-enkephalin-like peptides in adrenal venous plasma. Enkephaline-like peptides were extracted from plasma by acetone precipitation in acid, medium (0.01 N HCl). The extract was centrifuged and the supernatant was extracted 4 times with petroleum ether (4°C), (Acetone extent: Petroelum ether = 1:1). Residual acetone and petroleum ether were removed by a stream of N_2 and the remaining agueous solution was lyophilized. The residue was redissolved in H_2O, heated at 100°C for 10 min and centrifuged at 12,000 x g for 10 min. Aliquots of the supernatant were assayed radioimmunochemically using an antibody raised against met-enkephalin.

Major Findings: Secretion of met-enkephalin-like peptides from adrenal glands elicited by splanchnic nerve stimulation. The content of (met-)enkephaline-like peptides was highest in adrenal vein plasma (0.087 pmol/ml plasma), whereas plasma sampled from jugular vein, femoral vein and carotid artery contained less

(0.045 pmol/ml plasma). In anesthetized dogs electrical stimulation of the splanchnic nerve with increasing voltage from 5 to 30 V results in a voltage-dependent release of (met^5)-enkephalin-like peptides into the adrenal venous blood ranging from 150 to 300% over control. BioGel P-2 filtration of plasma extracts before and after stimulation revealed the presence of both high and low molecular weight peptides, though splanchnic nerve stimulation increases the plasma content of the low molecular weight species.

Measurement of the mean arterial blood pressure during splanchnic nerve stimulation resulted in an increase above control. However, if the dog received 2 daily injections of reserpine (0.5 mg/kg) which impairs the storage of catecholamines in the adrenal gland, stimulation of the splanchnic nerve caused a decrease of the mean arterial blood pressure.

Secretion of (met^5)-enkephaline-like peptides from adrenal glands elicited by morphine injection. Injection of morphine increases the amount of (met^5)-enkephalin-like peptides released into the adrenal venous plasma. Pretreatment of dogs with naloxone or prior transection of the splanchnic nerve were able to prevent this increase.

Significance to Biomedical Research and Institute Programs: Enkephalin-like peptides have been studied more extensively on their control of pain threshold, but the physiological function of these peptides does not appear to be limited to this effect. Since narcotic agents of clinical values are always showing respiratory=and vasodepressant effects and since the injection of morphine increases the content of (met^5)-enkephalin-like peptides in venous blood it appears important to reveal the action mechanism of these peptides on the cardiorespiratory system.

Proposed Course of Project: Future experiments are planned to reveal: (1) the nature of the receptor involved in regulating the release of the peptide hormones from the adrenal medulla, (2) the vasodilatory effect of the released peptides on the various peripheral vascular beds, (3) molecular characterization of the released peptides including purification, amino acid sequencing and production of specific antibodies directed toward these peptides.

Publications:

1. Costa, E., Guidotti, A., Hanbauer, I., Hexum, T.D., Saiani, L. and Yang, H.-Y.T.: Regulation of cholinergic transmission in adrenal medulla, In: Proceedings of the International Symposium on Cholinergic Mechanisms (ed., G.C. Pepen), Plenum Press, New York (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01885-02 HE

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pharmacological manipulation of CNS substance P systems (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Glen R. Hanson	Research Associate	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.8	OTHER: 0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A specific radioimmunoassay for substance P (SP) is being used to measure the effects of various pharmacological agents on the SP system in order to determine transmitter interactions and assign physiological roles to this neruopeptide.

618

Objectives: Substance P (SP) is postulated to be a neurotransmitter in the CNS. In order to study putative roles and physiological significance of SP function in the brain, changes in the levels of substance P-like immunoreactivity are determined in discrete CNS regions following pharmacological manipulation by agents which alter extrapyramidal and nociceptive systems.

Major Findings: Subacute treatment with dopamine (DA) antagonists, haloperidol and fluphenazine, reduced levels of SPLI in substantia nigra by 30-40%. The SPLI in other CNS regions were unaffected. The DA agonist apomorphine induced a 30% decrease of nigral SPLI in a specific manner, but did so 1 hr following a single injection of apomorphine. The two effects were characterized and shown to be dose-dependent (up to 1 mg/kg) and reversible with a rebound phenomena as part of the recovery pattern.

The subacute haloperidol effect was found to be mediated via a D_1-type receptor and could still be observed following the destruction of the DA nigrastriatal pathway, but not after the destruction of the striatonigral pathways.

The interaction between the SP and serotonergic systems in pons medulla was examined. A specific elevation of SPLI in the area of the dorsal raphe nucleus was observed following acute cocaine treatment. The effect appeared to be dose-dependent with some tolerance developing after chronic treatment. There was found to be a specific depression of SPLI also in the dorsal raphe which was induced by the serotonin precursor 5-HTP. The effects of both cocaine and 5-HTP on the levels of SPLI were attenuated when administered simultaneously. These changes in SPLI corresponded to inverse changes in 5-HT levels measured in pons-medulla.

The metabolism of SP is little understood, however, peptidase activity is thought to be responsible for its inactivation as a neurotransmitter. We have shown that intraventricular injections of SQ20881, a peptidase inhibitor, causes significant increases in the levels of SPLI in all brain areas examined. This elevation appeared to be dose-dependent in substantia nigra. However, SQ 20881 had differential effects on the SPLI in other tissues examined. In addition, two other peptidase inhibitors, SQ14225 and leupeptin were also observed to significantly elevate SPLI levels in substantia nigra and trigeminal nucleus. The agents might prove to be useful pharmacological tools to probe SP function.

Significance to Biomedical Research and Institute Programs: SP has been suggested to be important in the function of some neuronal systems. Thus, results indicate that (1) due to its excitatory influence on DA perikarya in substantia nigra, SP helps to modulate locomotor function and (2) SP is involved in sensory (particularly pain) transmission.

Little is known about the interaction of SP with other transmitters involved in locomotor and nociceptive function. Even less is known about the response of SP neurons to drugs which exert pharmacological influences on these neuronal systems. A better understanding of the involvement of SP in these complex neuronal networks would help to assign physiological and pathological roles to this neuropeptide and perhaps suggest new therapeutic approaches for the treatment of related neurological disorders.

Publications:

1. Hanson, G.R., and Lovenberg, W.: Elevation of CNS substance P-like immunoreactivity by protease inhibitors. J. Neurochem. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03501-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effects of Morphine on Calcium-Regulated Phosphorylation (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James P. O'Callaghan	Research Associate	HE NHLBI
OTHER:	Judith Juskevich	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Calcium (1-100 μM) caused a concentration and time-dependent increase in the phosphorylation of several synaptic cytosolic proteins. These effects of calcium were most apparent for proteins with molecular weights (M_r) of 50,000, 55,000 and 60,000. The administration of morphine, in vivo (3.0-30 mg/kg) caused a dose and time dependent increase in the phosphorylation of the 50,000, 55,000 and 60,000 molecular weight proteins compared to the values obtained from saline-treated controls. This effect of morphine was reversed by the specific narcotic antagonist, naloxone. Since calmodulin mediates calcium-dependent phosphorylation of synaptosomal cytosolic proteins, the effects of opiates on calcium-dependent protein phosphorylation may be through an action on this specific calcium binding protein.

Objectives: The biochemical signal generated by the binding of an opiate ligand to its specific receptor in neuronal membranes is translated into biochemical actions that alter neuronal function via effector systems. Since protein kinases and phosphoprotein phosphatases are localized in high concentration in synaptic membranes and synaptosomal cytosol and since specific phosphorylating proteins in the central nervous system may act as physiological effectors for a variety of regulatory agents, it is possible that neuronal phosphorylation systems may serve as mediators of the effects of endogenous and exogenous opiates.

The specific objectives of the present investigation were to evaluate the effects of the acute administration of morphine, the classic opiate analgesic, on the phosphorylation of specific synaptosomal cytosolic proteins obtained from rat striatal synaptosomes. Recently, it has been established that calcium ion regulates the phosphorylation of specific synaptosomal proteins, through an action on the specific calcium binding protein, calmodulin. Alterations, in brain levels of calcium as well as the binding of calcium brain membranes represent a possible mechanism through which the acute and chronic effects of opiates are mediated. Therefore, the current investigation has been confined to an examination of the effects of opiates on calcium regulated phosphorylation.

Methods:

Tissue preparation. Male, Wistar rats were killed by decapitation, the brains removed and the striata dissected within 30 seconds. A crude synaptosomal lysate was prepared from a striatal homogenate by differential centrifugation. This lysate was centrifuged at 140,000 x g for 90 minutes. The resulting supernatant fraction was used as a source of synaptosomal cytosol.

Phosphorylation assays. The net incorporation of phosphate into striatal synaptosomal cytosolic proteins was assayed at 30°C in a standard assay mixture containing (200 μl final volume) 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1.0 mM DTT and 5 μM (γ ^{32}P] ATP. Following a one minute preincubation of the assay mixture, the phosphorylation reaction was initiated by the addition of the synaptosomal cytosol. After one minute the reaction was terminated by solubilizing the cytosolic proteins in 100 μl of a smaple buffer containing 9% SDS, 0.03 M Tris-HCl buffer, pH 8.0, containing 6% mercaptoethanol, 3 mM EDTA and 27% sucrose. All samples were assayed at least in duplicate.

Gel Electrophoresis, Autoradiography and Microdensitometry. Proteins were resolved on SDS polyacrylamide slab gels. The acrylamide concentration was 6% and 10% in the stacking and resolving gels, respectively. Both stacking and resolving gels contained SDS at a final concentration of 0.1%. Following electrophoresis the gels were fixed and stained with 0.1% Coomassie blue R250 and 50% methanol, 10% acetic acid before drying under heat and vacuum. Autoradiography was carried out for a period of 1-2 days using Kodak RP X-ray film. The molecular weights of the protein bands were estimated from molecular weight standards. The incorporation of phosphate into specific protein bands was determined by microdensitometry of the autoradiographs using an EC 920 transmission microdensitometer. Protein phosphorylation was quantified from the densitormetric scans by integration of the areas under the peaks corresponding to specific protein bands.

Major Findings: Exogenous calcium (1.0-100 μM) stimulated the phosphorylation of at least 15 synaptosomal cytosolic proteins. Maximal stimulation due to calcium reached levels of as much as 25-fold those of control and was most apparent for proteins with molecular weights M_r of 50,000, 55,000, and 60,000. Only three major protein bands were phosphorylated in the absence of added calcium or in the presence of EGTA. Autoradiography revealed that morphine (3.0-30 mg/kg) caused a dose and time dependent increase in calcium-regulated protein phosphorylation compared to the autoradiographs obtained from saline-treated controls. These effects of morphine on calcium-regulated phosphorylation were reversed by the specific narcotic antagonist, naloxone. Microdensitometry of the autoradiographs confirmed that morphine enhanced calcium-stimulated phosphorylation. The maximal increase of 175% of control occurred at a dose of 30 mg/kg, 30 minutes following drug administration. In the absence of calcium, autoradiography revealed that morphine caused an increase in the phosphorylation of only one protein band (M_r, 89,000). This qualitative observation from the autoradiographs was also confirmed by quantitative microdensitometry. The effect of morphine on calcium independent phosphorylation was not found to be reversed by naloxone.

Significance to Biomedical Research and Institute Program: Although an involvement of opiate action in the dynamics of several neurotransmitter systems has been demonstrated, little progress has been made toward a delineation of the specific molecular mechanism(s) involved in the acute and chronic effects of narcotic analgesics. The phosphorylation of specific endogenous protein substrates represents one molecular mechanism through which numerous regulatory agents elicit specific biological responses. That a calcium-regulated phosphorylation system in neuronal cytosol may play an important role in synaptic function is indicated by the findings that the biosynthesis of neurotransmitters, e.g. tyrosine hydroxylase and tryptophan hydroxylase, are activated under phosphorylating conditions and can be regulated in a calcium dependent manner. Thus, calcium-regulated protein phosphorylation represents one possible locus of opiate action that may account for some of the acute and chronic effects of these drugs. Furthermore, calcium sensitive phosphorylation systems may be of physiological as well as pharmacological importance since the effects of the endogenous opiates, the endophrins, may also be mediated through an effect on calcium-regulated protein phosphorylation.

Proposed Course of Project: Several criteria must be met in order to establish whether a particular effect of an opiate compound is of the specific type characteristic of opiates as a class. These include: stereospecificity, blockade by a specific antagonist, tolerance, cross-tolerance and rank order of potency with other endogenous and exogenous opiates. We will attempt to establish if the effects of narcotic analgesics on calcium regulated phosphorylation meet the necessary criteria for a specific narcotic effect.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03502-02 HE

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biochemical Events Underlying the Fluctuations in cAMP Levels in Rat Pineal

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Staff Fellow	HE NHLBI
OTHER:	Larry Alphs	Research Associate	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The focus of this project was to describe in the rat pineal the sequale of biochemical events regulating cAMP levels following termination of β-receptor stimulation. Present results show that cAMP in rat pineals respond in a biphasic manner to diminished receptor stimulation. Levels of cAMP decreased significantly within 30 to 60 sec following exposure of dark-adapted animals to light or administration of propranolol (10 mg/kg i.v.). Within 5 min cAMP levels had significantly increased compared to 30 sec time points. While there were no changes in either cytoplasmic PDE or calmodulin levels, adenylate cyclase activity did increase at the same time that cAMP increased. Thus apparently cyclase activation could support the observed rise in cAMP levels. Present results are consistent with previous observations using dispersed pinealocytes and showing that diminished receptor stimulation leads to a fall in cAMP levels which precedes an abrupt drop in N-acetyltransferase activity. However, further investigation is necessary to link the change in cAMP with the fall in NAT.

PHS-6040
(Rev. 10-76)

Objectives: The pineal gland offers a unique opportunity to examine the biochemical mechanisms underlying short-term fluctuations in (1) the relationship between changes in cAMP content and the rapid fall of NAT levels, (2) the enzymatic mechanisms which subserve the observed changes in cAMP levels. Also the present investigation was performed using intact pineal glands rather than a culture system to provide data which more closely reflected in vivo conditions.

Methods: Male Sprague Dawley rats were used. The experimental paradigm consisted of exposing dark-adapted rats to light for various time periods (0.5, 1, 2, 4, 6 min) or administration (i.v.) of the drug propranolol. In this manner neuronal input to the pineal is diminished to such an extent that NAT preciptiously drops. The animals were either sacrificed by decapitation or placed in the microwave oven for 2 sec. Then the pineals were removed and hemogenized in the appropriate buffered solution and subsequently analyzed for either adenylate cyclase activity, phosphodiesterase activity, cytoplasmic calmodulin content, cAMP levels, or NAT activity.

Major Findings: Present results support the hypothesis that an abrupt decrease in cAMP levels is a necessary prerequisite for the rapid fall in NAT activity following diminished β-receptor stimulation. Under both experimental paradigms i.e., exposing dark-adapted animals to light or propranolol administration there was a recorded significant decrease in cAMP levels from control within 0.5-1 min. In both cases this decrease in cAMP preceded or coincided with the fall in NAT activity. Within 6 min, however, the levels of cAMP had begun to increase. In the light exposed animals the increase was approximately double control whereas in the propranolol-treated animals the cAMP had returned to control levels.

An analysis of the enzymes responsible for maintaining cAMP levels revealed some interesting findings. There were no changes in either PDE activity or levels of cytoplasmic calmodulin. Also there were no changes in adenylate cyclase (AC) activity analyzed within one min of light exposure. However, after 6 min AC activity had significantly increased above control levels. This change appeared to support the observed rise in cAMP levels also recorded at 6 min.

Significance to Biomedical Research and Institute Programs: Present results in combination with previous observations using dispersed pinealocytes are consistent with the fact that cAMP levels decrease before the abrupt drop in NAT activity. However, whether or not these two events are associated still remain an open question. Recruiting cAMP not only as the second messenger in the eventual induction of NAT but also in the maintenance of elevated levels of this enzyme would be in keeping with the known transduction of information from the CNS through the β-receptor within the pineal. However, further investigation is obviously necessary in order to link the change in cAMP with the fall in NAT.

The pineal is a useful model for understanding and characterizing the innervation of end-organ from a biochemical standpoint because the innervation is simple, anatomically well characterized, and accessable. Furthermore,

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the biochemical events occurring after neural stimulation of this system are dramatic. As such this model offers an almost ideal opportunity to characterize pre-and post-synaptic events of neural transduction. Results from analysis of this system might offer insights into the pharmacology and physiology of less well-defined systems in sympathetically innervated as well as into the central nervous system itself.

Proposed Course of Project: To further analyze and characterize the changes in adenylate cyclase which accompany the changes in cAMP levels.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03503-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of a Proenzyme I from Rat Brain (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Vivian S. Zabrenetzky	NIH Postdoctoral Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Donald M. Kuhn	Staff Fellow	HE NHLBI

COOPERATING UNITS (if any)

None

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Hypertension-Endocrine

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Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A cyclic nucleotide of independent kinase has been purified from rat brain supernatant. It phosphorylates, preferentially, histone as substrate in a magnesium dependent reaction. It is also calcium sensitive and in its non-trypsinized proenzyme form may contain the calcium binding protein calmodulin. It may also phosphorylate tryptophan hydroxylase in a calmodulin dependent manner.

PHS-6040
(Rev. 10-76)

Objectives: Because the kinase is calcium sensitive the presence of calmodulin in its structure will be determined. Tyrosine hydroxylase and tryptophan hydroxylase are activated by phosphorylations in a calcium dependent manner therefore the role of the protein kinase in activation of these enzymes will be determined.

Methods: The enzyme is purified through DEAE, Sephadex G-100 chromatography and isoelectric focusing. Polyacrylamide gel electrophoresis (PAGE) is used to determine the subunit composition of the enzyme. Fluphenazine-Sepharose affinity chromatography as well as activation of phosphodiesterase are used to determine the presence of calmodulin in the enzyme.

Major Findings: A highly purified protein kinase was obtained by the isolation procedure described above. A brief exposure to trypsin resulted in a 17-fold activation of the protein kinase. Either before or after proteolytic activation, the enzyme was essentially not stimulated by cyclic nucleotides (cAMP or cGMP). It shows optimal activity towards histone as substrate at the non-physiological level of 75 mM Mg^{++}. Purified enzyme which had been dialyzed against 0.5 M NH_4CO_3 or .025 M Tris (pH 7.5) was free of Ca^{++} and showed a two-fold enhancement of activity towards histone type II after the addition of 40 μM, $CaCl_2$ at 400 μM, $MgSO_4$. At 75 mM $MgSO_4$, the calcium stimulatory effect was not observed.

The purified enzyme caused a two-fold activation of calmodulin dependent phosphodiesterase (PDE). This activation of the PDE followed heat inactivation of the kinase and was calcium dependent. These findings correspond characteristics of other brain-calmodulin enzymes.

Purified, dialyzed and lyophilized enzyme was subjected to PAGE in 10% and 12% disc gels, (+) or (-) sodium dodecyl sulfate (SDS). The enzyme appeared as a single band in gels that were (+) or (-) 1% SDS. In order to determine the subunit composition of the enzyme, the effect of mercaptoethanol (ME) on its banding pattern was determined. The enzyme is activated by 30 mM ME and it was determined that as little as 1% ME caused the dissociation of the enzyme into at least 5 bands. The presence of trypsin or Ca^{++} can cause the appearance of an additional two bands, one of which is a low molecular weight protein.

Fluphenazine-linked Sepharose has a specific affinity for calmodulin in the presence of Ca^{++}. Preliminary experiments with the purified, dialyzed prokinase show that a fraction of the enzyme's activity is retained on the column in the presence of calcium and is released by the addition of the calcium chelator EGTA.

Significance to Biomedical Research and Institute Programs: It has long been known that calcium modulates neuronal transmission and that phosphorylation mediates this process. Since the prokinase is calcium sensitive and may contain the calcium binding protein calmodulin, the proenzyme may be the link between the two processes. It may provide this link by phosphorylating tryptophan hydroxylase and thereby regulate serotonin synthesis in a calcium dependent manner.

Since the psychotropic drug fluphenazine has been shown to bind a fraction of the enzyme, the effect of this drug on the enzyme's activity will be determined. The mechanism of action of this drug would thereby be elucidated.

Proposed Course of Project: The presence of calmodulin in the enzyme is being assayed by further gel studies and fluphenazine-affinity chromatography.

The activity of the enzyme as a modulator of catecholamine synthesis is being studied by the addition of tryptophan hydroxylase to the purified enzyme under phosphorylating conditions. The activation of the enzyme will be determined with special reference to its calcium dependent action.

The ability of psychotropic drugs (e.g., fluphenazine) to alter the enzyme will be determined by gel studies and enzyme activity profiles.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03504-02 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Stimulation of Rat Brain Tryptophan Hydroxylase by Polyelectrolytes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Mary Anne Meyer	Guest Worker	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

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Biochemical Pharmacology

INSTITUTE AND LOCATION

NHBLI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The activity of rat brain tryptophan hydroxylase (TH) is increased 2-fold by heparin and 4-fold by dextran sulfate. Hyaluronic acid, chondroitin sulfate, and dermatan sulfate as well as the unsulfated polymer dextran do not alter hydroxylase activity. Heparin and dextran sulfate decrease the apparent K_m of the enzyme for both substrates $6MPH_4$ and tryptophan as well as increasing the V_{max}. A variety of polyanions (DNA, glycogen, poly-d-and poly-ℓ-glutamic acid) have no effect on TH whereas salts [NaCl, KCl, $(NH_4)_2 SO_4$ and $MgSO_4$] inhibit the enzyme, indicating that the effects of heparin and dextran sulfate on TH are not mediated by increases in ionic strength per se. Several lines of data suggest that TH binds ionically to these polyelectrolytes: (1) The activation produced by heparin and dextran sulfate diminishes as the ionic strength of the assay medium increases, (2) TH binds to heparin substituted Sepharose 4B and is eluted by increasing the ionic strength of the eluant buffer, and (3) large molecular weight dextran sulfate (MW=500,000) dramatically shifts the elution profile of TH from a K_{av} of 0.41 to a K_{av} of 0.10 on a Sepharose/CL-6B column. These data suggest that binding of certain poly-electrolytes to TH induces a conformational change in the enzyme resulting in increased catalytic activity.

Objectives: The *in vitro* activity of tryptophan hydroxylase can be increased by a variety of substances including the membrane detergent sodium dodecyl sulfate (SDS), phospholipids (Hamon *et al.*, J. Neurochem. 28: 811, 1977) and ATP-Mg^{++} (Kuhn *et al.*, BBRC 82: 759, 1978). Upon close examination these seemingly different treatments may have a common effect on the enzyme. In fact, insight into the molecular mechanisms by which these treatments alter tryptophan hydroxylase can be gained from the results of experiments on a similar enzyme, tyrosine hydroxylase. For example, heparin, phospholipids, and certain biological membranes stimulate the activity of tyrosine hydroxylase. These structurally unrelated substances are all polyanions which apparently activate tyrosine hydroxylase via salt reversible, "electrostatic" interactions with the enzyme (Katz *et al.*, Biochem. Biophys. Acta. 429: 84, 1976). In an effort to more completely understnad how the activity of tryptophan hydroxylase is altered by polyanions, including SDS and phospholipids, the effects of various acidic mucopolysaccharides and the model polyelectrolyte dextran sulfate on tryptophan hydroxylase were investigated.

Methods: Male Sprague Dawley rats were decapitated and the mesencephalic tegmentum was rapidly dissected from the rest of the brain, frozen on dry ice and stored in liquid N_2. Tryptophan hydroxylase was assayed by the method of Friedman *et al.* (J. Biol. Chem. 247: 4165, 1972) as modified by Baumgarten *et al.* (J. Neurochem. 21: 251, 1973).

Major Findings: The major findings of this project were reported in detail in the last annual report. In furtherance of that report, the interaction of tryptophan hydroxylase with polyionic cellular organelles has been demonstrated (see Project report Z01 H1 01865-04 HE).

Significance to Biomedical Research and Institute Programs: These results indicate that tryptophan hydroxylase, like tyrosine hydroxylase, can be activated by its association with polyanionic molecular species. Heparin and other sulfated mucopolysaccharides are present in and transported along neuronal pathways; therefore, the alteration in tryptophan hydroxylase activity by polyanions may be a likely mode of regulation of this important enzyme *in vivo*. These results also contribute more generally to knowledge about the electronic and possible conformational requirements for maintaining optimal rates of tryptophan hydroxylation. The recent immunohistochemical demonstration of what appears to be an association of tryptophan hydroxylase with subcellular organelles further supports the suggestions that binding of tryptophan hydroxylase to cellular particles (perhaps with net anionic charges) leads to increases in enzyme activity.

Proposed Course of Project: Additional studies will be carried out to identify other cellular polyanionic substances which can activate tryptophan hydroxylase. Advantage will also be taken of the ability of tryptophan hydroxylase to bind to heparin and dextran SO_4 in the purification of the hydroxylase. Conformational changes in the enzyme structure will be studied in more detail (using a purer enzyme preparation) in an effort to assess the extent to which rapid changes in enzyme structure (in response to a variety of stimuli) lead to increases in activity and, in turn, increases in the neurotransmitter serotonin.

Finally, it was recently demonstrated that tyrosine hydroxylase can interact with tubulin. It will be of interest to determine if tryptophan hydroxylase can associate electrostatically with microtubules as does tyrosine hydroxylase.

Publications:

1. Kuhn, D.M., Meyer, M.A. and Lovenberg, W.: Activation or rat brain tryptophan hydroxylase by polyelectrolytes. Biochem. Pharmacol. 28: 3255-3260, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03505-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of Oxygen, Iron, and Sulfhydryl Groups in the Stabilization and Catalytic Activity of Tryptophan Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Belle Ruskin	Research Assistant	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Tryptophan hydroxylase appears to be an oxygen sensitive, iron requiring enzyme. Exposure of the crude enzyme to 100% O_2 for 30 min. at 37°C produces a 70% reduction in activity. Superoxide and hydroxyl radicals are not involved in the inactivation. There is no loss of activity at higher temperatures if the enzyme is kept under N_2. Activity can be recovered by the addition of thiols. Iron chelators can also strongly inhibit tryptophan hydroxylase, at least in part, by competitive inhibition for the substrates $6MPH_4$ and tryptophan. If the enzyme is exposed to 25°C in room air (20% O_2) for 24-48 hrs, a substantial loss of activity is observed. Activity can be largely recovered by incubating the enzyme (24 hrs) under anerobic conditions in the presence of dithiethreitol and iron at 25°C. Preliminary experiments have also suggested that the addition of inorganic sulfide produces an additional recovery of activity. Finally, sulfhydryl reagents strongly inhibit tryptophan hydroxylase. Taken together, these data suggest that tryptophan hydroxylase can exist in various activity states depending on the extent of oxidation or reduction of critical SH groups. The enzyme may be an iron sulfur protein.

PHS-6040
(Rev. 10-76)

Objectives: Tryptophan hydroxylase is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter serotonin. Progress in the determination of the physical and regulatory properties of this important enzyme has been slow, apparently as a result of the extreme lability of tryptophan hydroxylase. The primary objectives of these experiments were to determine the effects of sulfhydryl groups, iron and oxygen on the stability and catalytic activity of tryptophan hydroxylase. It is of particular interest to determine if tryptophan hydroxylase is an iron-sulfur protein.

Methods: Tryptophan hydroxylase was assayed in rat mesencephalic tegmental extracts by the method of Baumbarten et al. (J. Neurochem. 21: 251, 1973). For 0_2 and N_2 studies the enzyme was put into 15 x 100 glass tubes fitted with airtight stoppers. The enzyme solution is gassed through a 21 gauge needle and exhausted through another 21 gauge needle. Both needles were removed after 3 min of gas (0_2 or N_2) exchange.

Major Findings: Exposure of tryptophan hydroxylase to 100% 0_2 at 37°C results in rapid losses of activity. Under these conditions the enzyme has a half-life of 12-14 min. The loss of activity seen was prevented if 0_2 was replaced by N_2. Superoxide and hydroxyl radicals were not responsible for the loss of activity since the radical scavengers superoxide dismutase, catalase, inosine, mannitol, and histidine did not prevent 0_2 inactivation. The 0_2 induced inactivation could be prevented and reversed by a variety of reductants including dithiothreitol, ℓ, cysteine, ascorbate, mercaptoethanol and sodium borohydride.

The iron chelators o-phenanthroline, α, α-dipyridyl, and 8-hydroxyquinoline and desferioxamine are potent inhibitors of the hydroxylation reaction. It appears that the inhibition produced by these agents is mediated, at least in part, by competitive inhibition for the cosubstrates $6MPH_4$ and tryptophan. Chromatography of the enzyme on Sephadex G-25 after preincubation with o-phenanthroline does not completely reverse the inhibition, possibly indicating that there is residual o-phenanthroline still bound to the iron in the enzyme. The enzyme gradually recovers activity as the Fe-o-phenanthroline complex dissociates. A more potent and very specific iron chelator, deferoxamine, also inhibits tryptophan hydroxylation. The inhibition by iron chelators can be reversed by the addition of iron to the assay.

Reconstitution of enzyme activity can be successfully completed as described below. Enzyme inactivated by exposure to room air (20% 0_2) at 25°C for 24-48 hrs. has been successfully reconstituted by incubating the enzyme anaerobically at 25°C in the presence of 5 mM dithiothreitol and 50 μM ferrous ammonium sulfate for 24 hrs. The highest recovery of activity is obligatory to both substances. In a few experiments the inclusion of inorganic sulfide lead to a slightly greater recovery of activity. Enzyme inhibited by higher concentrations of 0_2 can be also completely reconstituted by anaerobic incubation in the presence of DTT and Fe^{2+}

Finally, the sulfhydryl reagents PCMB, iodoacetate, N-ethyl maleimide, and mercuric acetate each inhibited tryptophan hydroxylase. Mercuric acetate was the most potent inhibitor. The inhibition by sulfhydryl reagents can be rapidly reversed by dithiothreitol.

Significance to Biomedical Research and Institute Program: These studies have identified a few critical factors which are responsible for stabilization of tryptophan hydroxylase. As a direct result, more diligent attempts to purify the enzyme can now be made. Once accomplished, numerous studies on the physical, kinetic, and regulatory properties of the purified enzyme can be carried out. These types of studies have not been possible in the past because of the failure of investigators to achieve significant purification, ostensibly as a result of the extreme lability of tryptophan hydroxylase. These experiments have also suggested that tryptophan hydroxylase may be an iron or iron-sulfur protein, a fact which was implied over 15 years ago but not completely verified.

Proposed Course of Project: Experimentation will continue in the area of enyzme stabilization and the possibility of extraction and purification of tryptophan hydroxylase anerobically is being pursued. Additional kinetic studies are proposed to assess the roles of iron and sulfur in the catalytic activity of tryptophan hydroxylase. In addition to anaerobic chromatography, attempts to purify brain tryptophan hydroxylase by affinity chromatography will be carried out using a matrix with the pterin cofactor covalently bound to an agarose backbone. A second chromatographic procedure currently under investigation involves hydrophobic interaction chromatography on phenyl-Sepharose.

Publications:

1. Kuhn, D.M., Ruskin, B., and Lovenberg, W.: Tryptophan hydroxylase. The role of oxygen, iron, and sulfhydryl groups as determinants of stability and catalytic activity. J. Biological Chemistry 255: 4137-4143, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03507-02 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

α-Receptor Binding in Various Areas of Brain From Hypertensive Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Staff Fellow	HE NHLBI
OTHER:	Frank Douglas	Clinical Associate	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

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NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of the present project was to probe for the relationship between α-receptors in discrete areas of rat brain and blood pressure regulation. Specially bred WKY, SHR and SHR/SP rats were used for this investigation. The specific areas removed and analyzed were the hypothalamus, hippocampus, an area containing the locus coeruleus and the A_2 area containing the nucleus tractus solitarius. The ligands used for the analysis were the antagonist, WB4101 and the agonist clonidine. There were no differences in WB4101 or clonidine binding in any of the areas examined.

PHS-6040
(Rev. 10-76)

Objectives: The role of the central nervous system in the etiology of essential hypertension is at present unclear. There has, however, been some evidence suggesting a link between the noradrenergic system and hypertension. Over the last few years techniques have been developed whereby one can investigate certain functional characteristics of neuronal systems. One of these is the probing of receptors by the use of specific ligands. The purpose of the present project was to apply this technique to a recently bred hypertensive strain of rats.

Methods: Linearity with respect to protein concentration was confirmed in each region for both WB 4101 and clonidine binding. For the comparative analysis each ligand binding was analyzed at low concentrations (2 mM) while specific binding was displaced using 10 μM phentolamine.

Major Findings: Binding studies using the α-receptor agonist or antagonist revealed no differences in the extent of binding in any of the regions examined in the SHR rats.

Significance to Biomedical Research and Institute Programs: The potential role of altered receptor function in the CNS as factor in blood pressure regulation is an important unresolved question. The current studies, however, failed to reveal differences in the genetically hypertensive rats.

Proposed Course of Project: Perform an analysis using other radioligands specific for other receptors to determine their involvement in hypertension.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03508-02 HE

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Sympathetic Activity in the Rat Pineal

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Larry Alphs	Research Associate	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.5	0.5	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an initial group of experiments, pineal N-acetyltransferase activity (NAT) was measured after pretreatment of rats with phenoxybenzamine (POB). Since POB stimulated NAT activity in light-treated rats and since propranolol prevents the effect of POB, it is likely that POB's ability to prevent the light-mediated decrease in NAT activity is by way of stimulatory action of the drug at the pineal beta-receptor. In vivo experiments demonstrating that ganglionectomy and adrenalectomy do not prevent the action of POB suggest that this effect is produced by direct action of the drug on the pineal beta-receptor and not an indirect action mediated by another central or peripheral mechanism. Experiments demonstrating stimulatory activity of POB on NAT activity in cultured pineals confirm this suggestion. In a second series of experiments the role of cyclic AMP in the rapid light-mediated decrease in NAT activity was investigated. The decrease in NAT activity was consistently accompanied by a decrease in cAMP content after one minute of light exposure to dark-adapted rats. This was followed by a more marked rise in cAMP content after 4-6 minutes. These changes in cAMP were prevented by pretreating rats with POB and were in part mimicked by propranolol.

Objectives: These experiments were designed to understand those events involved in the neuroregulation of pineal NAT activity.

Methods: Male Sprague Dawley rats were used in these experiments. In those experiments investigating the role of cyclic nucleotides in NAT regulation special care was taken to ensure that the rats were maintained on a 12 hour light-12 hour dark lighting schedule. NAT was assayed by the method of Deguchi and Axelrod (Analyt. Biochem., 50: 174, 1972). Cyclic AMP was assayed by a radioimmunoassay adopted from Steiner et al. (J. Biol. Chem., 247: 1106, 1972).

Major Findings: Pineal N-acetyltransferase (NAT) activity was measured after pretreating rats with phenoxybenzamine (POB) (40 mg/kg) under a variety of lighting conditions. POB prevented both the light-mediated decrease in NAT activity and the decrease observed (even in rats left in the dark) at the onset of their customary light period. Rats administered POB and maintained in the light demonstrated increased pineal NAT activity 3-4 hours later. This effect of POB was greatly enhanced in superior cervical ganglionectomized rats, but was unaffected by adrenalectomy. The induction was blocked by propranolol suggesting that the effect is ultimately mediated by pineal beta-receptors.

Cultured pineals demonstrated elevated NAT activity when incubated with POB. This effect was also enhanced when pineals were obtained from rats which had been previously ganglionectomized. Initial experiments also suggest that pineals incubated with a combination of POB and norepinephrine (NE) produce an induction in NAT that is greater than that which is achieved with either drug alone.

These results suggest that POB stimulated pineal NAT activity directly by pinealocyte. This effect is apparently ultimately mediated by the pineal beta-receptor. POB itself may be working directly on the beta-receptor or possibly at a site that modifies the response to beta-adrenergic stimulation.

Measurement of pineal cAMP content after exposing dark-adapted rats to light for 1-6 min revealed that a decrease in NAT activity was accompanied by a decrease in cAMP content. This decrease in cAMP content was very transient and was followed by a marked increase in cAMP content. These changes in cAMP were reproduced when a decrease in NAT activity was precipitated with intravenous administration of propranolol except that no increase in cAMP content above control values was observed. This increase in cAMP corresponded temporally with an increase in adenylate cyclase activity.

The observation that a 50% decrease in pineal cAMP content accompanies the light-mediated decrease in NAT and that this decrease in cAMP is prevented when POB is used to prevent the light-mediated decrease in NAT, suggest that these two events may be linked. Thus, a decrease in cAMP may serve as a triggering mechanism for the decrease in NAT activity.

Significance to Biomedical Research and Institute Programs: This project seeks to use the pineal as a model system for understanding neuroregulation of post-synaptic biochemical processes. Because the changes in enzyme activity are

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both rapid and marked the pineal seems ideal for this type of research. By using this system in conjunction with a number of pharmacological agents not only can a more complete understanding of the neurotransmission within the sympathetic nervous system be achieved, but the mechanism of action of these drugs can also be more clearly elucidated--drug classes which are commonly used therapeutically. Finally, this work may also be expected to ultimately lead to a clearer understanding of the function of the pineal itself.

Proposed Course of the Project: Because the principal investigator plans to resume residency training elsewhere, this project will be terminated at the end of June 1980.

Publications:

1. Alphs, L., Lovenberg, W. and Heller, A.: Adrenergic regulation of the light mediated fall of acetyl coenzyme A: Arlamine N-acetyltransferase activity in the rat pineal. J. Neurochem. 34 (1): 83-90, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03509-02 HE

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cyclic AMP Specific, Calcium Independent Phosphodiesterase from a Murine Mastocytoma (Revised Title)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Krishnamoorthy Sankaran	Visiting Fellow	HE NHLBI
OTHER:	Donald Kuhn	Staff Fellow	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem Pharmacol	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.2	.2	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A cyclic AMP specific phosphodiesterase has been identified in a malignant tumor (P 815) of murine mast cells. The PDE is found primarily (85%) in the soluble fraction of the cell. The enzyme, purified approximately 10-fold by gel filtration, occurs in a single molecular and kinetic form (low K_m), and is apparently not dependent on calcium and calmodulin for optimal activity. Although cGMP is hydrolyzed at only 4% of the rate of cAMP hydrolysis, this cyclic nucleotide inhibits cAMP PDE activity by 50-60% at a concentration 2.5 μM. The mast cell PDE activity also displays anomalous behavior on gel filtration and sucrose gradient centrifugation. The Stokes radius was determined by gel filtration to be 54.4 Å and the $s_{20,w}$ was determined by gradient centrifugation to be 2.73S. Using these values a molecular weight of 61,000 and a frictional ratio of 1.93 were calculated. This PDE is apparently an asymmetric enzyme with novel molecular and regulatory properties.

Objectives: Multiple forms of cyclic nucleotide phosphodiesterase (PDE) have been identified in extracts of many mammalian tissues. These forms differ in their kinetic behavior, substrate specificity and response to various effectors and seem to exist in different ratios in various type cells. At least one form of these phosphodiesterases can be activated by calmodulin, an endogenous thermostable protein. Interaction of this enzyme with calmodulin and calcium results in a decrease in the K_m of the enzyme for substrate and an increase in V_{max}. However, a complete definition of the elements involved in the regulation of these various forms has not been achieved. Therefore, we sought to determine some of the primary molecular and regulatory properties of the mast cell PDE.

Methods: Male Balb/c x DBA mice were used and neoplastic (P 815) murine mast cells were harvested and transplanted as previously described (Z01 HL 03509-01 HE). Phosphodiesterase activity was assayed by the method of Filburn and Karn (Analyt. Biochem., 1973, 52, 505-516).

Major Findings: The cAMP hydrolyzing activity in mast cells was confined almost exclusively (85%-90%) to the soluble fraction of the cell. The PDE occurs in a single molecular and kinetic (low K_m) form and is apparently not dependent on either calcium or calmodulin for optimum activity. The Stokes radius was determined by gel filtration to be 54.4 Å and the $s_{20,w}$ was determined by gradient centrifugation to be 2.73S. Using these values, a molecular weight of 61,000 and a frictional ratio of 1.93 were calculated.

Significance to Biomedical and Research Institute Programs: The foregoing results with the mast cell PDE are significant in that they represent the first demonstration of a PDE enzyme which exists in a tissue as a single molecular and kinetic (high affinity) form. Furthermore, the enzyme is substrate specific for cAMP and is apparently not dependent on either calcium or calmodulin for optimal activity. Additional studies are necessary to determine if this PDE is the prototypical catalytic unit of many other PDE enzymes.

It has been suggested that malignancy is often associated with low effective levels of cAMP. The cytosol fractions of hepatoma cells are deficient in cAMP binding sites and a shift in the elution profile of liver protein kinases, the activities of which were associated with cAMP were reported in Yoshida ascites tumor bearing rats. The high relative specific activity of PDE, resulting in low levels of cAMP (Preliminary experiments revealed that the concentration of cAMP in cell extracts is 5.3 nM and the concentration of cGMP is 5.2 nM), may therefore have some bearing on various aspects of altered cellular metabolism in the malignant mast cells.

Proposed Course of Project: Studies are underway to purify the mast cell PDE to homogeneity. Once purified, more direct physical methods will be used to determine molecular weight and other properties of the PDE. In addition, attempts will also be made to produce antibodies to the purified PDE.

In an attempt to learn more about the relationship of cellular malignancy to the activation of PDE, cells will be assayed for PDE at various stages of malignancy so that a correlation can be made between total enzyme activity and altered cellular proliferation. With antibodies, it can be determined whether the enzyme is activated or if additional enzyme molecules have been synthesized. Finally, attempts will be made to ascertain if calmodulin is in fact a subunit of the mast cell PDE. We will take advantage of new affinity chromatographic methods which can specifically isolate calmodulin containing proteins.

Publications:

1. Sankaran, K., Kuhn, D.M., and Lovenberg, W.: Cyclic AMP specific, calcium independent phosphodiesterases from a malignant murine mast cell tumor. Biochem. Biophys. Res. Commun., 1979, 89, 793-799.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03510-01 HE

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Calcium-regulated protein phosphorylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James P. O'Callaghan	Research Associate	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Lawrence Dunn	Normal Volunteer	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.5	OTHER: 0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Calcium stimulated the endogenous phosphorylation of several synaptosomal cytosolic proteins. The effects of calcium were both concentration and time dependent and were most apparent for proteins with molecular weights (M_r) of 50,000, 55,000 and 60,000. Exogenous calcium (1.0-100 μM) enhanced the net incorporation of phosphate into protein by as much as 23-fold. The antipsychotic, fluphenazine (1.0-100 μM), caused a concentration dependent decrease in calcium stimulated protein phosphorylation. When the heat-stable calcium binding protein, calmodulin, was removed from synaptosomal cytosol by affinity chromatography on fluphenazine-sepharose, calcium-stimulated protein phosphorylation was abolished. Responsiveness to calcium could be stored by the addition of calmodulin to the phosphorylation assay. These results indicate that calcium dependent protein kinases are of major importance in regulating the phosphorylation of specific cytosolic proteins in neuronal tissue.

Objectives: Calcium ion, perhaps through the activation of a specific calcium dependent protein kinase, has been found to regulate the phosphorylation of specific proteins found in preparations of synaptic plasma membranes, synaptic vesicle membranes, cerebral cortical slices, synaptosomes, and synaptosomal lysates. Recent evidence indicates that the calcium dependent phosphorylation of synaptic plasma membranes and synaptic vesicles requires the heat-stable calcium binding protein, calmodulin. Since the calmodulin-regulated protein phosphorylation of synaptic vesicle proteins have been linked to the release of neurotransmitter from isolated vesicles, this protein phosphorylation system may play an important role in neuronal function. Although the calcium-calmodulin activated protein kinases and associated substrates in neuronal membranes have been identified and partially purified, specific calcium and/or calmodulin requiring protein kinases and associated substrates have not been identified in neuronal cytosol.

The specific objectives of the present investigation were to determine the extent of phosphoprotein substrates in neuronal cytosol that were phosphorylated in a calcium dependent manner. Since the soluble fraction of neuronal tissue (synaptosomes) is known to have a high concentration of calmodulin, the effects of calcium on protein phosphorylation were likely to be mediated through protein kinases that require calmodulin as a coenzyme. Therefore, we determined if the calcium regulated phosphorylation of synaptosomal cytosolic proteins satisfied the criteria for a calmodulin mediated effect.

Methods:

Tissue preparation. Male, Wistar rats were killed by decapitation, the brains removed and the striata dissected within 30 seconds. A crude synaptosomal lysate was prepared from a striatal homogenate by differential centrifugation. This lysate was centrifuged at 140,000 x g for 90 minutes. The resulting supernatant fraction was used as a source of synaptosomal cytosol.

Phosphorylation assays. The net incorporation of phosphate into striatal synaptosomal cytosolic proteins was assayed at 30°C in a standard assay mixture containing (200 μl final volume(50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1.0 mM DTT and 5 μM [γ ^{32}P] ATP. Following a one minute preincubation of the assay mixture, the phosphorylation reaction was initiated by the addition of the synaptosomal cytosol. After one minute the reaction was terminated by solubilizing the cytosol proteins in 100 μl of a sample buffer containing 9% SDS, 0.03 Tris-HCl buffer, pH 8.0, containing 6% Mercaptoethanol, 3 mM EDTA and 27% sucrose. All samples were assayed at least in duplicate.

Removal of calmodulin from synaptosomal cytosol. Aliquots of synaptosomal cytosol were subjected to affinity chromatography on fluphenazine-sepharose columns (0.5 x 5.0 cm) in order to remove endogenous calmodulin from the cytosol. Following chromatography, the eluates were divided into aliquots and the protein values were adjusted to 100 μg per sample. Calmodulin previously prepared by affinity chromatography, was then added to half of the samples and all tubes were assayed for phosphorylation as described above.

Gel Electrophoresis, Autoradiography and Microdensitometry. Proteins were resolved on SDS polyacrylamide slab gels. The acrylamide concentration was 6% and 10% in the stacking and resolving gels, respectively. Both stacking and resolving gels contained SDS at a final concentration of 0.1%. Following electrophoresis the gels were fixed and stained with 0.1% Coomassie blue R250 and 50% methanol, 10% acetic acid before drying under heat and vacuum. Autoradiography was carried out for a period of 1-2 days using Kodak RP x-ray film. The molecular weights of the protein bands were estimated from molecular weight standards. The incorporation of phosphate into specific protein bands was determined by microdensitometry of the autoradiographs using an EC 290 transmission microdensitometer. Protein phosphorylation was quantified from the densitometric scans by integration of the areas under the peaks corresponding to specific protein bands.

Major Findings: Calcium (1.0-100 μM) stimulated the phosphorylation of several specific synaptosomal cytosolic proteins. These effects were both concentration and time dependent and were most apparent for proteins with molecular weights (M_r) of 50,000, 55,000 and 60,000. Maximal stimulation of phosphorylation by calcium was as great as 25-fold above control levels. In the absence of added calcium, the calcium chelator, EGTA, did not lower the phosphorylation of any protein below control levels, however, EGTA was completely effective in blocking calcium-stimulated phosphorylation. A number of antipsychotic drugs bind to calmodulin with high affinity in a calcium dependent fashion. We exploited this property of antipsychotics in order to examine the role of calmodulin in the calcium stimulated phosphorylation of synaptosomal cytosolic proteins. Two approaches were chosen. In the first, the antipsychotic, fluphenazine, was added to the phosphorylation assay to serve as a calmodulin antagonist. In the second, fluphenazine was linked to sepharose in order to serve as an affinity matrix for the removal of calmodulin from synaptosomal cytosol. The addition of fluphenazine (1.0-50 μM) to the phosphorylation assay caused a concentration dependent decrease in calcium stimulated protein phosphorylation. As little as 25 μM fluphenazine reduced the calcium stimulated phosphorylation to levels equal to those observed in the absence of calcium. When synaptosomal cytosol was subjected to affinity chromatography on a fluphenazine-sepharose matrix it was found that this procedure was effective in removing endogenous calmodulin and resulted in an abolition of calcium stimulated phosphorylation. Responsiveness to calcium could be restored by the addition of calmodulin (0.5 μg) to the phosphorylation assay.

Significance to Biomedical Research and Institute Program: Although specific phosphorylated proteins found in neuronal membranes have been implicated as physiological effectors for the diverse actions of a variety of intracellular regulatory agents, including calcium, little attention has been given to the possibility that proteins in the cytosol compartment may function in a similar capacity. Our findings that physiological levels of calcium stimulate the phosphorylation of specific cytosolic proteins in a calmodulin dependent fashion supports the hypothesis that calcium serves to transmit within the cytosol by functioning as a second messenger when bound to specific soluble proteins such as calmodulin. That a calcium-regulated phosphorylation system in neuronal cytosol may play an important role in synaptic function is indicated by the

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e.g., tyrosine hydroxylase and tryptophan hydroxylase, are activated under phosphorylating conditions and can be regulated in a calcium dependent manner.

Proposed Course of Project: Specific calcium-calmodulin regulated protein kinases and the associated phosphoprotein substrates in neuronal cytosol will be identified and purified in an attempt to correlate the degree of phosphorylation with the functional state of these specific proteins.

Publications:

1. O'Callaghan, J.P., Dunn, L.A., and Lovenberg, W.: Calcium regulated phosphorylation in synaptosomal cytosol: Dependence on calmodulin. Proc. Nat'l Acad. Sci. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03511-01 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Role of the Brain Serotonergic Neuronal System in Blood Pressure Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Donald M. Kuhn	Staff Fellow	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	William A. Wolf	Research Associate	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.8	0.7	0.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Electrical stimulation of the dorsal or median raphe nucleus in anesthetized rats produces a transient increase in arterial blood pressure. Heart rate and respiratory rate do not appear to change systematically after stimulation. Transections rostral or caudal to the raphe nucleus or electrolytic lesions of the nucleus linearis abolishes the pressor response to stimulation. Depletion of brain serotonin with PCPA significantly attenuates the pressor effect of both dorsal and median raphe stimulation. Fluoxetine, a specific serotonin uptake inhibitor, prolongs the duration of the raphe pressor effect and slightly increases its magnitude. Injections of the serotonin antagonist BOL into the anterior hypothalamus area significantly attenuates the dorsal raphe pressor effect whereas treatment of rats with the sympathetic postganglionic blocking agent bretylium prevents the stimulation induced pressor effect. Taken together these data suggest that stimulation of the ascending serotonergic neuronal system produces a phasic pressor effect which is mediated, at least in part, by synaptic serotonin.

PHS-6040
(Rev. 10-76)

Objectives: The contributions of the central nervous system to the regulation of arterial blood pressure have been investigated in some detail. However, the role of discrete neurochemical systems, such as the catecholaminergic and especially the serotonergic systems, have not been studied extensively. The purpose of this project was to assess the role of the ascending serotonergic neuronal system in regulation of blood pressure using techniques of electrical stimulation of the brain and pharmacological manipulation of the serotonergic system.

Methods: Male Sprague-Dawley rats were used. Rats were anesthetized with chloropent and one femoral artery and one femoral vein were cannulated. The subjects were then mounted in a Kopf sterotaxic instrument for stimulation. Twisted bipolar electrodes (0.25 mm diameter) were aimed at the dorsal or median raphe and electrical current was delivered via a WPI dual channel pulse stimulator using stimulus isolation units to ensure delivery of a constant current stimulus. The following parameters were varied at the indicated values: frequency (1,10,25,50Hz), current (100-500μA), pulse duration (0.1-1.0msec). Alternating biphasic squarewave pulses were delivered in trains lasting for 5 sec. Blood pressure was measured with a Statham P23ID transducer and recorded on a Sanborn Polygraph. After stimulation experiments brains were fixed in formaldehyde and electrode placement was verified histologically.

Major Findings: Stimulation of either the dorsal or median raphe nucleus produced pressor effects which were proportional to the frequency and current parameters of electrical stimulation. Increases in blood pressure as large as 80 mm Hg were observed at 50 μA, 50 Hz. Heart rate and respiratory rate fluctuated slightly, but did not change systematically after raphe stimulation. The anatomical specificity of the pressor effect was confirmed by functional-anatomical studies. Pressor effects were only observed if the electrodes were in either raphe nucleus. Transections of the brain rostral or caudal to the raphe nuclei abolished the pressor response to raphe stimulation. Electrolytic lesions of the nucleus linearis, a locus of ascending serotonergic tracts, also abolished the pressor response to stimulation.

Depletion of brain serotonin with PCPA significantly lessened the pressor response to raphe stimulation. Inhibition of uptake of serotonin with the drug fluoxetine slightly increased the magnitude of the pressor response and significantly prolonged its duration. Injections of the serotonergic antagonist BOL directly into the area of the anterior hypothalamus also significantly attenuated the pressor response to raphe stimulation. Finally, the sympathetic postganglionic blocking agent bretylium markedly attenuated the raphe pressor response.

Significance to Biomedical Research and Institute Programs : These results establish clearly that the brain serotonergic neuronal system constitutes an important central pressor network. These data also integrate well with a growing body of evidence which has recognized the potential importance of brain serotonin in the regulation of the cardiovascular system. Furthermore, these experiments have opened a new avenue of investigation into the pharmacologic and brain physiologic regulation of blood pressure.

Proposed Course of Project: The following experiments will be undertaken to delineate the anatomical and neurochemical determinants of hypertension within the serotonergic neuronal system. Some of these experiments are in progress:

1. Serotonin will be microinjected into various brain areas while recording blood pressure. The site of most importance appears to be the anterior hypothalamus.

2. Genetically hypertensive rats and their controls will be stimulated as described above and blood pressure and heart rate will be monitored.

3. Discrete chemical lesions of the brain serotonin system will be induced with the neurotoxin 5,7-DHT and the development of renal hypertension (DOC-salt) will be monitored.

Publications:

1. Kuhn, D.M., Wolf, W.A., and Lovenberg, W.: Review of the role of the central serotonergic neuronal system in blood pressure regulation. Hypertension 2: 243-255, 1980.

2. Kuhn, D.M., Wolf, W.A., and Lovenberg, W.: Pressor effects of electrical stimulation of the dorsal and median raphe nuclei in anesthetized rats. J. Pharmacol. Exp. Ther., in press, 1980.

3. Kuhn, D.M. and Lovenberg, W.: Vaso- and psychoactive substances in foods. In: Nutritional Toxicology, Nathcock, J.N. (ed)., Academic Press, 1980 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03512-01 HE

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Characterization of Changes in Rat Adrenal TH Activity

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Larry Alphs	Research Associate	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI
	Eleanor Bruckwick	Research Assistant	HE NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.75	.25	.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Kinetic analysis of adrenal tyrosine hydroxylase (TH) with respect to varying concentrations of 6-methyltetrahydropterin cofactor demonstrated two distinct forms of the enzyme-one with a relatively low cofactor affinity and a high V_{max} and another with a 100-fold greater cofactor affinity and a low V_{max}. Initial Scatchard analysis by computer suggests that phosphorylation of adrenal TH produces qualitatively distinct changes in the kinetics of these 2 forms of TH. Passing a crude adrenal homogenate over Dowex-50 seems to convert the enzyme to single kinetic form with intermediate K_m's and V_{max}'s.

Objectives: The purpose of this work was to characterize the kinetic changes in rat adrenal TH activity that occur when TH is activated by in vitro phosphorylation or when catecholamines are removed from a crude homogenate by passing it over ion exchange resin.

Methods: Male Sprague-Dawley rats were used. Rats were decapitated and adrenals were rapidly removed and assayed for TH activity by the method of Lerner et al. (Neurochemical Research, 3, 641 1978). Computer analysis was used to evaluate the kinetic parameters for V_{max} and K_m using a modified program written by Faden and Rodbard (SCATFIT, 1976).

Major Findings:

1) Adrenal TH activity is observed to have 2 major kinetic forms with respect to the cofactor 6-methyltetrahydropterin. One form has a relatively low cofactor affinity and a high V_{max} and another has a 100-fold greater affinity for this cofactor and a lower V_{max}.

2) Phosphorylating the enzyme in vitro is seen to increase the K_m of the high affinity form while markedly increasing its V_{max} and to decrease the K_m of the low affinity form with little change in V_{max}.

3) Changes produced by phosphorylation are not as readily apparent at the in vitro pH optimum of TH as at higher, possibly more physiologic, pH's (6.4-6.8).

4) Passing a crude adrenal homogenate over Dowlex-50 produces a single kinetic form of TH with intermediate values for V_{max} and K_m.

Significance To Biomedical Research and Institute Programs: This project seeks to investigate the regulation of TH in the adrenal as a model for understanding mechanisms of control of the adrenergic and dopaminergic nervous systems. The evidence of 2 kinetic forms of TH is highly significant, as it suggests that previous data investigating only a narrow range of cofactor concentrations have, in reality, been observation of the sum of changes in these two forms. A more detailed analysis of our current work with in vitro activation of TH with phosphorylation and endogenous activation is likely to give both a more detailed and accurate understanding of the mechanisms of control of this important adrenergic enzyme. These findings also have potential for deepening our understanding of diseases as varied as schizophrenia, Parkinson's disease and others involving the adrenergic or dopaminergic nervous system.

Proposed Course of Project: Future experiments are planned to repeat the present data to allow for a more detailed statistical analysis of activation mechanisms of TH. We plan to expand this work to look at the control of striatal TH which preliminary experiments also demonstrate to have 2 kinetic forms with respect to cofactor.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03513-01 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Correlation of Different Neurotransmitter Systems in Rats After Treatment with Cocaine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Sikta Pradhan	Staff Fellow	HE NHLBI
OTHER:	Glen Hanson	Research Associate	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The acute administration of cocaine (15-30 mg/kg) consistantly increases substance P in dorsal raphe with a corresponding decrease in 5-HT levels. When rats are treated with 5-hydroxytryptophan (5-HTP) along with the decarboxylase inhibitor, RO4-4602, 5-HT content in PM increases with a significant decrease in SP level in dorsal raphe. But when cocaine, 5-HTP and RO4-4602 are administered simultaneously, the changes in 5-HT and SP levels are attenuated. These results suggest that there is a specific interaction between the neurotransmitters substance P and 5-HT in the dorsal raphe area.

PHS-6040

Objectives: Certain psychomotor stimulatants such as cocaine, amphetamine and methyl phenidate appears to have serotonergic effects in central nervous system. It has been demonstrated in a series of studies by Pradhan et al that behavioral effects of these drugs such as motor hyperactivity, sterotypy and increase in self-stimulation behavior can be correlated with the drug-induced increase in dopamine (DA) levels in the caudate nucleus (CN) and diencephalon-midbrain (DM) and decrease in serotonin (5-HT) levels in the DM and Pons-medulla (PM). Furthermore, in cocaine-treated rats pretreatment with the 5-HT precursor, 5-hydroxytryptophan (5-HTP) along with a decarboxylase inhibitor (RO4-4602) at a dose that replenished the previously reduced levels of 5-HT, also reduced the elevated levels of DA in the respective brain area as well as inhibited the induced behavioral effects.

The objective of this study was therefore to test for the dynamic interaction if any between the SP and 5-HT systems in certain CNS areas by using a psychomotor stimulant such as cocaine and 5-HTP, the serotonin precursor as pharmacological tools and the sensitive radioimmunoassay technique for estimation of SP.

Major Findings: The interrelationship of substance P-like immunoreactivity (SPLI) in rat dorsal raphe nucleus to the brain content of 5-hydroxytryptamine (5-HT) was investigated in animals treated with the serotonin-active drug cocaine. Following the acute administration of this compound (15-30 mg/kg) there was consistently a specific increase of SPLI in dorsal raphe which was accompanied by a significant decrease of 5-HT levels in pons medulla. Treatment of rats with the 5-HT precursor, 5-hydroxytryptophan (5-HTP) along with the decarboxylase inhibitor, RO4-4602, caused increments in 5-HT content with concurrent decreases in dorsal raphe SPLI. When cocaine and 5-HTP were administered simultaneously the changes in serotonin and SPLI were attenuated suggesting that each drug antagonized the effect of the other. These data support a specific interdependence of substance P and 5-HT in the dorsal raphe nucleus.

Proposed Course of Project: Current literature indicates that there is a close relationship between dopamine, adenylate cyclase and membrane-bound form of calmodulin. Our preliminary studies on the effects of cocaine on $[^3H]$-spiroperidol binding activity show that the apparent b_{max} of $[^3H]$-spiroperidol binding in membrane fraction of striatal homogenate is increased 20 min after injection of cocaine (20-30 mg/kg) with a little or no change in the affinity of the labelled ligand to the recognition site. Also, under similar conditions of cocaine treatment, a significant increase in calmodulin content of crude synaptic membrane fraction was observed with no change in soluble calmodulin pool (cytosol). Hence, at this state, it will be important to establish whether the increment in membrane-bound calmodulin elicited by cocaine can be associated with the activation or to supersensitivity of striatal adenylate cyclase to dopamine.

Publications:

1. Hanbauer , I., Pradhan, S. and Yang, H-Y.T.: Role of calmodulin in dopaminergic transmission. Annl. Rept. New York, 1980 (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03514-01 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Heat Stable Cytosolic Factors Affecting Synaptic Membrane Phosphorylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Judith Juskevich	NIH Postdoctoral Fellow	HE NHLBI
OTHER:	James P. O'Callaghan	Research Associate	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine Branch

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.7	0.7	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The specific phosphorylated proteins found in neuronal membranes have been implicated as physiological effectors for the diverse actions of the second messenger candiates, cyclic AMP and calcium. It has been shown that calcium-dependent phosphorylation of neuronal membranes require the heat stable calcium-binding protein, calmodulin. Since the calmodulin-regulated protein phosphorylation of synaptic vesicle proteins has been linked to release of neurotransmitter from isolated vesicles, this phosphorylation system may play an important role in synaptic function. Other factors may be present in the cytosol compartment that are important in determining the steady or dynamic phosphorylation states of specific synaptic membrane proteins. We present evidence for the existence of a heat-stable factor in synaptosomal cytosol that stimulates phosphorylation of a specific synaptic membrane protein in a calcium- and calmodulin-independent fashion.

Objectives: Synaptic function may be affected by the phosphorylation state of specific proteins found in the synaptic membranes. Therefore, it is important to study the factors that regulate protein kinase activity. We are studying factors found in synaptosomal cytosol to determine their effect on the activity of membrane-bound protein kinases in an attempt to determine the factors involved in functional regulation of membrane phosphorylation.

Methods: Synaptosomes were isolated from a crude P_2 fraction of rat whole brain by flotation on a discontinuous ficoll gradient. A synaptosomal lysate was prepared by exposure of the synaptosomal fraction to hypotonic conditions at basic pH. The synaptosomal membranes were obtained from the lysate by centrifugation and were used to prepare a synaptic plasma membrane fraction by discontinuous sucrose gradient centrifugation. The synaptosomal cytosol was heated, centrifuged and the supernatant used as a source of calmodulin.

The phosphorylation of the synaptic plasma membrane fraction was assayed at 30°C in a mixture (200 μl final volume) containing 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1.0 mM DTT, 5 μM [γ ^{32}P] ATP, in the absence or presence of 300 μM $CaCl_2$. The reaction was initiated with the addition of membrane. After 15 seconds the reaction was terminated and membrane proteins were solubilized by the addition of 100 μl of an electrophoresis sample buffer.

Synaptic membranes were then resolved on SDS polyacrylamide slab gels. The phosphorylation of specific synaptic proteins was determined by autoradiography.

Major Findings: When synaptic membranes were incubated in the presence of γ ^{32}P ATP, several proteins were phosphorylated. Cyclic AMP, in the presence or absence of calcium stimulated the phosphorylation of two proteins. The addition of heated synaptosomal cytosol results in a markedly enhanced phosphorylation of several proteins. Calcium was required for the effects of heated cytosol on the phosphorylation of all proteins with the exception of a protein with an apparent molecular weight of 85,000 (85 K band). That the incorporation of phosphate into the 85 K band was independent of calcium was confirmed by addition of EGTA.

The pattern of the calcium-calmodulin-regulated phosphorylation duplicated the pattern seen in the presence of heated cytosol with the exception that the phosphorylation of the 85 K band was only marginally enhanced.

In the presence of fluphenazine, calmodulin did not affect the incorporation of phosphate into any protein, with or without the addition of calcium to the assay mixture. However, in the presence of fluphenazine, the addition of heated cytosol restored the phosphorylation of the 85 K band in a calcium-independent manner.

When calmodulin is removed from heated cytosol, by means of affinity chromatography, the remaining effluent stimulated the phosphorylation of the 85 K band in a calcium-independent manner, whereas the incorporation of phosphate into most other proteins remained unchanged.

The results presented in this report demonstrate the existence of a heat-stable factor in synaptosomal cytosol that stimulates the incorporation of phosphate into a specific synaptic membrane protein. The effects of this factor on phosphorylation do not require calcium and can be distinguished from the calmodulin- and cyclic AMP-regulated systems.

Significance to Biomedical Research and Institute Programs: Phosphorylation of specific proteins found in neuronal membranes may be involved in modulating synaptic function. Phosphorylated proteins have been implicated as physiological effectors for the diverse actions of a variety of intracellular agents. Therefore, studying the regulation of protein phosphorylation may result in a greater understanding of the mechanisms involved in the regulation of synaptic function.

Proposed Course of Project: Further work will be done to purify and characterize this calcium-calmodulin and c-AMP independent factor which stimulates protein phosphorylation. Some of the methods which will be employed include fractionation on DEAE columns, molecular seive chromatography and sucrose density gradients.

There has been some indication that a heat stable inhibitory factor also exists. Therefore, we will attempt to determine the presence of an inhibitory factor in heated cytosol and also determine its role in the regulation of protein phosphorylation.

Publications:

1. O'Callaghan, J.P., Juskevich, J., and Lovenberg, W.: Stimulation of synaptic membrane phosphorylation by a calcium and calmodulin independent heat stable cytosol factor. Biochem. Biophys. Res. Comm. 95: 82-89, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03515-01 HE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Analysis of Fluctuations in Rat Pineal Tyrosine Hydroxylase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Leonard Miller	Guest Worker	HE NHLBI
OTHER:	Sikta Pradhan	Research Associate	HE NHLBI
	Mary Mikus	Summer Worker	HE NHLBI
	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Hypertension-Endocrine

SECTION
Biochemical Pharmacology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.6	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project was to make a comparative analysis of tyrosine hydroxylase in rat pineals from light-adapted and dark-adapted rats. In this gland both the rate of utilization and concentration of N.E. increased when animals are in the dark phase of a 24 hr light/dark cycle. Thus, a comparative analysis would reveal alterations in tyrosine hydroxylase activity which could possibly account for the observed increase in neurotransmitter synthesis. Using subsaturating cofactor concentrations T.H. activity in dark-adpated rats was 60% > in appropriate light control animals. Further kinetic analysis showed that the K_m of cofactor using tissue from dark-adapted animals was 0.8 ± 0.1 mM which was significantly lower than a K_m value of 1.4 ± 0.2 mM for light-adapted animals. Thus, the rate of norepinephrine synthesis in vivo could increase due to an alteration in the enzyme molecule.

PHS-6040
(Rev. 10-76)

Objectives: To analyze how a system would respond within the limits of an endogenously controlled system to a normal physiological stimulus.

Methods: Male Sprague Dawley rats were kept on a reversed lighting schedule lights on 6 p.m. - 6 a.m. Rats were sacrificed by decapitation and pineals removed and homogenized in phosphate buffer containing tritox-100. Tyrosine hydroxylase activity was determined by measuring the amount of tritiated water formed during the conversion of tritiated tyrosine to DOPA.

Findings: All detectable tyrosine hydroxylase activity in the pineal appears associated with synaptic contacts. An analysis of T.H. at subsaturating cofactor concentrations in dark-adapted rats revealed activity which was 60% higher than in appropriate light-control animals. Further kinetic analysis showed that the K_m of cofactor for the dark-adapted animals was 0.8 ± 0.1 which was significantly lower than a K_m of 1.4 ± 0.2 for the light-adapted animals.

Significance to Biomedical Research and Institute Programs: The changes recorded in this study were within the limits of an endogenously controlled and fluctuating system. Therefore, the results give us an indication not only as to how the system will respond endogenously to meet the needs for increased transmitter synthesis, but also the degree to which the system will change. Most important is that the recorded events were initiated totally by a normal physiological stimulus.

Proposed Course of Project: Study has been completed.

Publications:

1. Miller, L.P., Stier, M., and Lovenberg, W.: Evidence of presence of N-acetyltransferase in rat retinal tissue. Comp. Biochem. and Physiol. (In press).

2. Miller, L.P. and Lovenberg, W.: A comparative analysis of the effect of actinomycin-D, α-amanitin and cordycepin on new RNA synthesis and N-acetyltransferase induction by isoproterenol in cultured rat pineals. Comp. Biochem. and Physiol. (In press).

660

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03516-01 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Biosynthesis, Distribution and Biological Role of Substance P

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mei Lie Swenberg	Research Chemist	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.4	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Substance P is a neuropeptide which serves as a neurotransmitter. Little is known of its synthesis or metabolism or the physiologic role that its neurons serve. We have found this neurotransmitter to be involved in the modulation of the dopaminergic system. Other studies in our laboratory have shown that it is apparently metabolized by an enzyme similar to the angiotensin converting enzyme. Preliminary studies have also shown it to be present in adrenal chromaffin granules. A system has been established to measure its rate of synthesis following intraventricular injection of radioactive amino acids.

PHS-6040
(Rev. 10-76)

Objectives: Substance P (SP) appears to be a transmitter of major importance in mammalian brain. It has been postulated to serve as a role in pain recognition, cardiovascular control (baroreflex), and as a modulator of the nigral-striatal dopaminergic system. The current study encompasses several aspects of SP function.

(1) Mechanisms underlying its specific biosynthesis will be investigated. These will include characterization of the precursor protein and a study of amino acid incorporation into SP. Attempts will be made to examine amino acid incorporation in vivo and in primary cultures of brain cells.

(2) Because of the potential role of substance P in cardiovascular control, systems for measuring SP in human sera will be devised and levels will be measured in hypertensive and normal subjects. Studies on distribution and content will also be done on genetically hypertensive rats.

(3) The SP content and subcellular distribution in the adrenal medulla will be determined. The potential role of this organ as a source of circulating SP will be determined.

(4) A new radioimmunoassay will be established and antibodies will be raised in rabbits following injection of a SP-protein conjugate.

Methods: Substance P is measured by a standard radioimmunoassay with Try-8-SP labelled with iodine as the standard. The antibodies for this assay were donated by Dr. William Campbell. New Antibodies have been generated in rabbits following repeated injections of a SP-bovine serum albunum conjugate. Short-term tissue culture will be carried out with brain cells from various areas. e.g., substansia nigra, cordate, hypothalamus, cerebellum and both short- and long-term primary tissue culture of mid brain and hypothalamus of embryonic brain at different stages of gestation. Amino acid incorporation into SP will be measured by an immunochemical method. In addition to immunoprecipitation, newly synthesized SP will be identified by HPLC and electrophoresis. Synthesis in vivo will be examined following parenteral or intraventricular administration of radioactive amino acids. Ribosomes will be isolated from brain regions rich in SP and attempt to release a SP precursor will be made.

Major Findings: This project has been recently initiated. A strong titer of antibodies directed against SP has been generated in 2 of 5 rabbits injected with the SP conjugate . With tracer amounts of I-125 labelled SP a 1 to 10,000 dilution of the serum binds 40-50 SO of the SP.

With an established RIA the SP content of adrenal medullary cells in culture was examined. These cells contained moderate amounts (120 pg/mg protein) of SP which in part is localized in the chromaffin vesicles. Additional studies on content and distribution of SP in adrenal tissue are currently in progress. In the course of this study we also observed that adrenal cortex contains significant amounts of SP(200 pg/mg protein). Its role in this tissue is unknown.

Preliminary studies with administration of radioactive amino acids suggest that it will be possible to study the synthesis of SP in rat brain.

Significance to Biomedical Research and Institute Programs: Substance P appears to be a major neurotransmitter that participates in many physiologic control systems in the CNS. Among these may be the baroreflex. An understanding of the synthesis and role of SP is essential for furthering our knowledge on cardiovascular control.

Proposed Course of Project: The immediate objectives are to establish reliable systems for measuring synthetic rates of SP. Future studies will include characterization of the SP precursor and a definition of its physiologic roles.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03517-01 HE

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Genetical Aspects in the Development of Cardiac Hypertrophy in Rats

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Hisao Tanase	Guest Worker	HE NHLBI
OTHER:	Walter Lovenberg	Chief, Sect. Biochem. Pharmacol.	HE NHLBI

COOPERATING UNITS (if any)

Small Animal Resources Section, Veterinary Resources Branch, Division of Resources Branch

LAB/BRANCH

Hypertension-Endocrine

SECTION

Biochemical Pharmacology

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.0	1.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The strain differences of heart and aorta weight in fifteen strains of rats were investigated for characterizing the cardiac hypertrophy from the genetic aspect. A significant strain difference in the heart weight was observed at ten weeks of age, and the degree of genetic determination was 67.0 ± 5.8%. The distribution of heart weight was divided into hypertensive and normotensive groups. In the hypertensive group, the heart weight increased in proportion to the blood pressure levels. On the other hand, there was no relationship between heart weight and blood pressure level in the normotensive group in spite of large strain difference of heart weight. These data indicated that the cardiac hypertrophy in rat is regulated by genetic factors in addition to blood pressure. A similar result was obtained with the aortic weight. However, the effect of genetic factors was less important for aorta weight than for heart weight.

PHS-6040
(Rev. 10-76)

Objectives: Cardiac hypertrophy is often associated with sustained hypertension in man. It is generally assumed that the increased work load on the heart in these patients is the primary causative factor. With the availability at the NIH of a large number of inbred rat strains that exhibit a broad spectrum of basal blood pressure values, it appeared feasible to analyze the relation between heart size and blood pressure as well as other factors. We, therfore, undertook a detailed analysis of blood pressure, weight of heart, aorta and total body, as well as other parameters of cardiac hypertrophy in 15 strains of imbred rats including the spontaneously hypertensive rats.

Methods: Animals were obtained from the NIH small animal production unit. After arrival in the laboratory they were maintained under standard conditions and their blood pressures were measured repeatedly over the course of 2 weeks by tail cuff plethesomography. At ten weeks of age the animals body weight blood pressure and heart rate were recorded and the animals sacrificed. The heart and aorta were removed by a standard dissection technique and were weighed. The external and internal diameter of the heart and the ventricle wall thickness was measured.

Major Findings: As has been reported previously these 15 strains of imbred rats show a large range of resting systolic blood pressures at this age. The lowest value being 118 ± 1 mm Hg for the MNR/N strain and the highest being 187 ± 2 for the stroke-prone spontaneously hypertensive rats (SHR-SP/N). Of interest is the confirmation that the Osborn-Mendel (OM/N) strain is apparently slightly hypertensive (154 ± 1 mm Hg). Significant differences in the growth of the strains were also observed.

The total heart weights range from 558 ± 12 mg in Wistar Kyoto WKY/N to 872 ± 10 in the SHR. It is of interest that the strain exhibiting the highest pressure, the SHR-SP has a heart weight of this age of only 709 ± 23 mg. However when the smaller body size of this strain is accounted for, the relative heart size in the SHR-SP was the largest of any strain. The correlation between cardiac size and blood pressure is not striking. The same is true for aorta weight. Analysis of cardiac dimensions shows a positive correlation between heart weight and external diameter and ventricle wall thickness. There is a negative correlation between heart weight and internal diameter. The data accumulated is sufficient for a variance analysis which indicated genetic factors other than the stress of hypertension accounted for about 67% of the determinants for heart size.

Significance to Biomedical Research and Institute Programs: Cardiac hypertrophy is a serious clinical problem associated in part with sustained hypertension. The current study provides information suggesting that factors other than blood pressure have an important role in determining heart size. The fact that genetic factors play such an important role in determining heart size may have an impact on how we view cardiac hypertrophy.

Proposed Course of Project: In the coming year, two aspects of this study will be pursued. (1) An effort will be made to distinguish between hypertrophy and hyperplasia in the heart of the various imbred strains, and (2) We will attempt to determine the genetics of heart size by preparing crosses of some of the imbred strains and doing the same physical analysis as reported above.

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1979 THROUGH SEPTEMBER 30, 1980

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding of renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that control transport.

Isolated segments of renal tubules

In order to understand kidneys on a cellular and molecular level the functions of the different types of epithelial cells must be identified. Progress in this direction has relied heavily on the direct study of individual nephron segments. Each nephron segment has a different cell morphology and function. An important method (which originated in this laboratory) for directly studying the nephron segments is to dissect them and perfuse them individually in vitro. The findings, during the past year, using this method are as follows:

1. Sodium and potassium-activated ATPase (Na-K-ATPase) in isolated segments of rabbit renal tubules

Na-K-ATPase is the active transporter for sodium. Garg and Burg have developed a sensitive and reliable method for measuring Na-K-APTase in isolated nephron segments, based on the method previously described in this laboratory by Czaczkes et al. (1979). Using the modified method, they found that the enzyme level was highest in proximal convoluted tubules, distal convoluted tubules, medullary thick ascending limbs, and distal convoluted tubules. It was lower in proximal straight tubules, cortical thick ascending limbs and collecting ducts, and no activity was found in thin descending and ascending limbs of Henle's loop. The level of Na-K-ATPase in the different nephron segments correlated (r=.94) with the previously measured rates of sodium transport in the segments. Treatment of rabbits with deoxycorticosterone acetate (DOCA) caused Na-K-ATPase activity in cortical collecting ducts to increase about 6-fold without comparable changes in the other segments. Low salt diet had a similar, but smaller effect. Thus, the increase in sodium transport previously demonstrated in the cortical collecting duct following chronic mineralocorticoid stimulation, involves an increase in the Na-K-ATPase and, therefore, in the number of active sodium transport sites in that segment.

2 Control of sodium transport by renal tubules

Knepper and Burg have continued to investigate the sites and mechanisms of control of sodium transport by the nephron. Previously, they showed that chronic administration of pharmacologic doses of DOCA to rabbits in vivo resulted in increased fluid absorption and cell height in proximal straight tubules studied in vitro. Now they have tested whether endogenous mineralocorticoids have a similar direct physiologic effect. Rabbits were given a low sodium diet to increase circulating levels of endogenous aldosterone. Proximal straight tubules did not differ from untreated controls in the rate of fluid absorption or cell height. These experiments, therefore, provide no evidence that mineralocorticoids exert an important physiological influence on proximal sodium and water absorption. Further, although DOCA alone caused fluid absorption and cell height to increase, this did not occur when the DOCA was administered during sodium restriction. Therefore the increases in fluid absorption and cell dimensions following DOCA administration most likely result secondarily from changes in extracellular fluid volume, glomerular filtration rate, or potassium balance.

Although distal convoluted tubules and connecting tubules have high levels of Na-K-ATPase and presumably transport relatively large amounts of sodium, there have been no direct studies of their sodium transport in vitro. The reason is that these segments are short and difficult to isolate. Almeida and Burg have initiated study of the segments. They are beginning by measuring transepithelial voltage and resistance which are electrophysiological correlates of sodium transport.

3. Acidification and bicarbonate transport transport by rabbit cortical collecting ducts.

Final adjustments in the urine pH are dependent on the transport of bicarbonate and hydrogen ion in the distal nephron segments. Our previous work on one of these segments, the cortical collecting duct, showed that in vitro it either secreted or absorbed bicarbonate depending on the previous acid-base status of the animals from which the tubules were obtained. Boyer and Burg have further examined the bicarbonate secretory process. They found that, when they replaced chloride in the tubule fluid by sulfate, bicarbonate secretion was inhibited without any effect on bicarbonate absorption. Therefore, absorption and secretion are separate processes, subject to separate controls. It has long been known that intravenous infusion of sulfate results in replacement of chloride by sulfate in the urine and greatly augments urinary acidity. The interpretation of this result had been that sulfate affects sodium absorption in the distal tubule and that the alteration in sodium transport somehow increased hydrogen ion secretion. This interpretation has been a principal basis for the current theory that sodium transport is linked to and controls acidification in the distal tubule. There was no direct evidence for the theory, however, and it must now be reconsidered since the inhibition of bicarbonate secretion by sulfate observed in the present experiments provides an alternate explanation for the findings which does not involve sodium transport.

Atkins and Burg have developed a microelectrode suitable for measuring pH in small amounts of collected tubule fluid. They are using this device to examine the mechanism of acidification in proximal straight tubules and cortical collecting ducts.

Cell culture of kidney and other urinary epithelia

Although the technique of perfusing kidney tubules in vitro has provided an overall description of their transport properties, it has been difficult to extend the studies to subcellular and molecular levels. Chemical and physical methods for studying transport require much larger amounts of homogeneous tissue than are present in single tubules. Dr. Handler and his colleagues have been using culture of epithelial cells to overcome this difficulty.

Steele has developed a technique for culturing cells on thin collagen membranes. The advantages of his new method are that it not only permits direct visualization of the cells, but also nondestructive measurement of transepithelial electric potential, resistance, and short-circuit current. Burg, Green, Handler, and Steele have applied this technique to primary culture of rabbit nephron segments. Cells derived from thick ascending limbs produced an epithelium with electric potential oriented apical surface positive, as in the native tissue. The investigators are attempting to establish a line derived from these cells and further define its characteristics, as well as to apply the technique to other nephron segments and animal species.

Watlington and Handler have been characterizing nuclear aldosterone receptors in A6 cells, an established cell line derived from toad kidney. They measured number of binding sites, affinities and kinetics of competition by other steroids. One of the classes of aldosterone receptors had an apparent K_D of 10^{-8}M corresponding to the level of aldosterone that stimulates short-circuit current in these cells. Preston and Handler found that incubation of A6 cells with aldosterone increased specific binding of ^{3}H-ouabain, evidence that aldosterone increased the number of active sodium transport sites. This effect was blocked by amiloride, a drug that inhibits the initial step in sodium transport, namely sodium entry into the epithelial cells. Therefore, the increase in the number of sodium transporters probably is not a direct effect of the hormone. Instead, aldosterone increases sodium entry into the cells by an amiloride-sensitive mechanism, and it is the additional supply of sodium to the transport sites that causes their proliferation. Aldosterone also increases certain cellular proteins which could well be components of the sodium transporter (see last years annual report). If so, the synthesis of the new proteins should be blocked by amiloride, which is being tested.

Roy, Preston, and Handler studied receptors for vasopressin in $LLCPK_{1L}$ cells. This cell line (derived from pig kidney) grows in a totally defined medium. When serum or insulin was added to the growth medium the number of receptors for vasopressin increased without a change in their affinity, and there was a parallel increase in vasopressin-stimulation of adenylate cyclase. It had been dissappointing that some line of cells, such as those derived from toad bladders and kidney, had many of the characteristics of vasopressin-stimulated tissues, but did not respond to the hormone. Steele and Handler have now found that the response of the cells depends on the nature of the support on which they are grown. The investigators noted marked changes in A6 cells grown on collagen films. Those grown on the films developed active cilia, whereas no ciliary activity had been apparent in cells grown on solid supports. Most interesting, only the modified cells responded to vasopressin by increasing their cyclic AMP and the transport of sodium. Thus, vasopressin action can now be studied in the cells, and the reason for the previous lack of vasopressin action can be analyzed.

Dragsten (NCI) and Handler used fluorescent labels to study molecular mobility in the cell membranes of the epithelia formed by A6 cells. They found that intrinsic membrane glycoproteins in both the apical and basolateral plasma membranes were immobile. After the epithelium was disrupted by chelation, however, the glycoproteins moved all around the cells. Presumably, there are some cellular substructures that immobilize such membrane proteins only while the cells are organized in an epithelium, but not when the epithelium is converted into single cells. In contrast to glycoproteins, the lipids diffused readily within the plasma membranes. Certain types of labelled lipids moved readily from the apical to basolateral side of the cells (or vice versa) past the tight junction, but other types of lipids did not. The investigators are now analyzing what distinguishes the lipids that diffuse past the tight junctions from those that do not.

Turner is developing methods to prepare plasma membrane vesicles from epithelial cell cultures. In order to monitor the isolation of basal or apical plasma membranes, the membranes are identified by their characteristic enzymes and other markers. To date plasma membrane vesicles have been successfully prepared from two cell lines, A6 and LLC-PKI. The uptake of sodium by the vesicles from A6 cells was sodium concentration-dependent and inhibited by amiloride. The uptake of glucose by the vesicles from LLC-PK$_1$ was sodium-dependent and inhibited by phlorizin. Both sets of experiments demonstrate the feasibility of studying specific transport systems from the cultured cells in isolated membrane vesicles. Turner plans to study the systems already developed in more detail and to attempt to prepare vesicles from other cell lines. Such vesicles will provide a convenient starting material for characterization, isolation, and reconstitution of the transport molecules.

Transport of D-glucose by proximal tubule brush border membranes

D-glucose is actively reabsorbed from the urine in the renal proximal tubule. The active step in this process is D-glucose co-transport with sodium into the cells across the lumen brush border membrane. Turner is studying this process in membrane vesicles since, in addition to its obvious physiological significance, the brush border membrane D-glucose transporter is a useful model system for studying the mechanism of co-transport processes in general.

(1) Phlorizin binding studies

Phlorizin is a potent competitive inhibitor of renal D-glucose reabsorption. It competes with glucose for binding sites on the glucose transporters, but is not itself transported. Turner and Silverman found that the binding of phlorizin and sodium to dog renal cortical brush border membrane vesicles occurred via a random rather than an ordered process and that the stoichiometry of binding is one sodium ion per phlorizin molecule. Binding constants and other kinetic binding parameters for the interaction of phlorizin with its receptor were determined. Since the binding of glucose and sodium to the transporter is a random process, models of the transporter which require ordered binding (e.g. certain types of pores) are incorrect.

(2) Heterogeneity of the glucose transporter

Turner and Moran found that proximal tubule brush border membrane vesicles prepared from different regions of the kidney have different glucose transport properties. Preparations from the outer cortex had a low affinity ($K_m \sim 5mM$) for glucose while preparations from the outer medulla had a higher affinity ($K_m \sim 0.5mM$). The differences correlate well with the known distribution and characteristics of glucose transport along the proximal tubule. The earliest part of the proximal tubule (predominant in the outer cortex) transports glucose rapidly but does not establish large glucose concentration differences. The last part of the proximal tubule (predominant in the outer medulla) transports less glucose, but establishes large concentration differences, virtually eliminating glucose from the urine. Apparently the glucose transporters differ between those segments correspondingly. Differences in the nature of the sodium dependence and in the specificity for glucose analogs were also found. More detailed investigations of glucose transport kinetics, phlorizin binding properties and sodium-glucose stoichiometry will be carried out.

(3) Theoretical models of coupled transport systems

In order better to understand possible mechanisms of coupled transport and to design appropriate experiments to distinguish between them, Turner has analyzed the theoretical properties of a family of co-transport models of the carrier-type. The most general model allows the substrate molecule and its co-transported cation to bind in a random fashion at either membrane face. Various sub-models include ordered binding schemes such as the case where substrate binding necessarily preceeds cation binding and pore-type models where the order of binding is reversed at the two membrane faces. It can be shown that the various transport models can be distinguished from one another on the basis of a series of relatively simple kinetic measurements. Rejection criteria for carrier-type models in general are also being derived.

Necturus gallbladder epithelium

The cellular aspects of epithelial transport can be studied directly by making measurements in individual cells and in the spaces between them. Since mammalian cells generally are very small, such direct studies are difficult and there are artifacts due to cell damage. Therefore, Spring has been using the very large gallbladder cells of *Necturus* for this purpose.

(1) Lateral intercellular space osmolality

Coble and Spring are attempting to measure the osmolality of the fluid in the lateral intercellular spaces, since this is a crucial variable in fluid transport by epithelial tissues. The spaces are very small and the fluid within them cannot reliably be sampled by conventional means such as micropipets. The investigators are, therefore, using an optical method, microscope interferometry, to measure the refractive index of the fluid in the spaces. Refractive index and osmolality are proportional in salt solutions, and the method theoretically allows measurements of sodium chloride solution osmolality to within 4 mOsm.

(2) Hydraulic water permeability of the epithelial cell membranes.

Persson and Spring have measured this key factor in epithelial fluid transport by a new method. They determined cell volume by light microscopy before and after rapid alterations of the osmolality of the bathing solutions. The rate of cell swelling or shrinkage was used to calculate the water permeability of the cell membranes. The water permeability of both the apical and basolateral cell membranes is very high. Given such a high cellular water permeability, fluid most likely is absorbed through the epithelial cells rather than between them, and only very small osmotic pressures within the epithelium are required to drive the process.

(3) Cell volume regulation

Persson and Spring observed that changes in cell volume, caused by alterations in solution osmolality, were in time compensated for by the cells. This "volume regulation" also occurs in other cells, in which it is known to involve the gain or loss of cell solutes in response to the initial cell volume change. Fisher and Spring are now measuring intracellular electrolytes in *Necturus* gallbladder with ion-specific microelectrodes to find out which solutes are involved in volume regulation by the tissue.

Regulation of red blood cell volume

Kregenow has pioneered studies of cell volume regulation, using nucleated avian and amphibian erythrocytes as model systems. Siebens and Kregenow have now further studied the mechanism responsible for restoration of cell volume by *Amphiuma* red cells shrunk in hypertonic media. They observed that the cells swelled back towards their normal volume by taking up sodium salts, followed by osmosis of water. The energy for the process comes from the transmembrane concentration difference for sodium which normally is low in the cells. During the volume regulation process, sodium entered the cells by a special transporter, which was inhibited by amiloride. The transporter was found to be a sodium-proton exchanger. Therefore, the cells would tend to become alkaline during this process, except that the erythrocyte anion exchanger compensates by facilitating cellular uptake of chloride in exchange for bicarbonate or hydroxyl which are extruded. The net result is NaCl uptake by the cells. When the investigators specifically blocked the anion exchanger with SITS, chloride did not enter the cells along with sodium and there was an increase in cell pH during the swelling.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01217-05 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Epithelial fluid transport and morphology

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Kenneth R. Spring, DMD, Ph.D., LKEM/NHLBI

Other: Bo-Eric Persson, M.D., Ph.D., Visiting Fellow, LKEM/NHLBI

COOPERATING UNITS (if any)
Section on Theoretical Biophysics, NHLBI
Television Engineering Section, NIH

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION
Electrolyte Transport

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/2	0

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Epithelium cell volume is determined rapidly during changes in bathing solution osmolality and the rate of change of cell volume is used to calculate the hydraulic water permeability of the cell membranes. The measured permeability of the cell membranes to water is sufficiently high that normal fluid reabsorption may occur across the cell response to small osmotic gradients.

PHS-6040
(Rev. 10-76)

Objectives

The primary goal of this investigation is elucidation of the mechanism of fluid absorption by epithelia. The methods employed represent a unique blend of optical and electrical techniques developed specifically for this investigation. We use an on-line, real time system for measuring the size and shape of the cells. Based on changes in cell volume we are able to determine the hydraulic water permeability of the cell membranes.

Methods

The gallbladder of the amphibian Necturus maculosus is mounted in a chamber designed to allow the continuous perfusion of both surfaces of the tissue, measurement of epithelial electrical properties, variation in transepithelial hydrostatic pressure, and rapid alteration of perfusate composition. The chamber is placed on the stage of a Leitz inverted microscope. Position and focus of the microscope are monitored by observation of the preparation during measurements with the aid of a television camera. The video images are stored on a disc recorder and later analyzed by tracing cell outlines. Cell size and shape are determined from video records obtained during alteration in perfusate composition or other experimental manipulations.

Major Findings

The hydraulic water permeabilites of the apical and basolateral cell membranes have been determined by measurement of the rate of change of cell volume after alteration in bathing solution osmolality. The values obtained were: $1.1x10^{-3}$ cm/sec Osm for the apical cell membrane and $2.4x10^{3}$ cm/sec Osm for the basolateral cell membrane. Such high water permeabilities are consistent with the transcellular flow of water across this epithelium. Only small osmotic gradients (less than 5mOsm) are required across the cell membranes to produce fluid absorption at the rates normally observed in Ringer solution. Volume regulation, the return of cell volume towards its control value, was observed under all conditions. Ouabain caused cell swelling which could be blocked by removal of mucosal NaCl.

Proposed Course

Additional experiments to determine the mechanism of cell volume regulation are in progress. We will utilize a variety of inhibitors and ion substitutions to identify the mechanism of cell volume regulation as well as to quantitate the solute flows involved in such regulation.

Publications

1. K. R. Spring and B.E. Persson. Quantitative light microscopy and epithelial function, in Epithelial Ion and Water Transport, ed. A. D. C. Macknight and J. Leader. Raven Press, NY. 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO1 HL 01219-04 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Tissue culture of epithelial cells from the urinary tract

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: J. S. Handler, M. D., Hd., Sec. on Membrane Metabolism, LKEM/NHLBI

Other:
M. B. Burg, M. D., Chief, LKEM/NHLBI
F. Perkins, M.D., Guest Worker, LKEM/NHLBI
C. Watlington, M. D., Guest Worker, LKEM/NHLBI
A. S. Preston, Chemist, LKEM/NHLBI
N. Green, Chemist, LKEM/NHLBI
J. Johnson, M. D., Medical Officer, WRAIR
A. Moran, Ph.D., Visiting Scientist, LKEM/NHLBI
R. E. Steele, Ph.D., TD/NHLBI
C. Roy, M. D., Guest Worker (Special Consultant), LKEM/NHLBI

COOPERATING UNITS (if any)
Lab. of Technical Development, NHLBI
Dept. of Nephrology, WRAIR
Lab. of Theoretical Biology, NCI

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.4	5.4	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose is to characterize existing lines of cells and develop new lines of cells that in culture perform transepithelial transport of interest to the nephrologist. We are attempting to start new lines of cells beginning with primary culture from microdissected segments of the nephron. Cells of lines cultured from the toad urinary bladder transport sodium actively. The rate of transport is stimulated by aldosterone and by cyclic AMP. The latter stimulates permeability to urea but has no effect on permeability to water.

PHS-6040
(Rev. 10-76)

Objectives

The continued purpose of this project is the utilization of epithelia formed in culture to better understand transport and its control in naturally occurring epithelia. The need for this approach and its possible advantages have been discussed in previous reports.

Methods

See previous reports and the annual report of R. Steele, LTD, NHLBI and the annual report of P. Dragsten, LTB, NCI.

Major Findings

There is continued slow progress in work with primary culture derived from rabbit kidney. In order to study the transport properties of primary cultures, dissected thick ascending limb segments have been grown directly on a collagen membrane (see report of R. Steele for methods). Although success has not been achieved routinely, several preparations have grown out to form a confluent epithelium that developed a transepithelial electrical potential difference with apical surface positive, as in vivo. Conditions for culture have been improved to the point that subculturing cells grown on plastic petri dishes is now more successful. Similar cultures were also derived from amphibian kidney.

The two epithelial cell lines derived from toad urinary bladder (see last year's report)have been characterized further In addition to stimulating sodium transport, cAMP increases permeability to urea (as in the toad urinary bladder) but in contrast to its effect on the intact toad bladder, cAMP does not increase permeability to water in the cultures. The relation between aldosterone concentration and short-circuit current of the cultured cells is like that of the intact bladder. The effect of thyroid hormone differs between the two lines. In one (TB-6c), as in the toad urinary bladder, incubation with thyroid hormone blocks the short-circuit current response to aldosterone. In the other line (TB-M) thyroid hormone has no effect on the response to aldosterone.

Continuous lines of kidney-derived epithelial cells have been used to study properties of the plasma membrane of epithelia. These results are summarized in the annual report of P. Dragsten, LTB, NCI.

We have obtained a strain of cells (LLC-PK$_{1L}$) derived from LLC-PK$_1$ cells (which are derived from porcine kidney) that grow in a completely defined, serum-free medium. The cells form a confluent epithelium with domes, an indication of transepithelial transport. LLC-PK_{1L} cells have vasopressin-sensitive adenylate cyclase activity, but only about 5% as much as LLC-PK_1 cells. Receptors for vasopressin, assessed by the specific binding of ^{3}H-lysine vasopressin, are increased in number when cells are grown in the defined medium to which serum or insulin has been added. The effects of serum and insulin are additive and reversible. There is an increase in vasopressin-sensitive adenylate cyclase that parallels the increase in vasopressin receptors. Kinetic analysis indicates that the increased binding is the result of more binding sites rather than a change in affinity for binding vasopressin.

Significance

The various epithelial properties retained by cells in culture make them particularly suitable for study not otherwise possible, e.g. - microelectrode impalement and measurement of the effect of prolonged exposure to hormones or drugs. The effect of insulin on receptors for vaospressin is the first demonstration of that relationship.

Proposed course

Efforts to grow primary cultures from defined segments of the nephron will be continued exploring other species and culture conditions. Receptors for aldosterone will be assessed in the cell lines derived from toad urinary bladder in order to see whether thyroid hormone blocks the effect of aldosterone at the receptor level. The transport properties of LLC-PK_{1L} cells will be defined and then, since the cells grow in a defined medium the influence of hormones and related factors on transport will be evaluated. In collaboration with Drs. Spring and Fisher of LKEM, intracellular ion activities will be measured by impaling the epithelial cells with appropriate microelectrodes. This will facilitate estimation of the driving forces for transport and should elucidate the mechanism of effect of hormones on transport.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01224-04 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Control of sodium and potassium transport by isolated rabbit tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Mark Knepper, M.D., Ph.D., Research Assoc., LKEM/NHLBI

Other: M. B. Burg, M. D., Chief, LKEM/NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Kindey & Electrolyte Metabolism

SECTION
Electrolyte Transport

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER: 0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project is designed to delineate sites and mechanisms of hormonal control of renal sodium and potassium transport. Previous studies with isolated perfused outer cortical proximal straight tubules from rabbits demonstrated that chronic administration of deoxycorticosterone acetate (DOCA) increased fluid absorption and cell volume. Further experiments to determine whether these are direct effects of DOCA revealed: (a) maintenance on a low sodium diet did not increase fluid absorption or cell volume. b) DOCA administration to rabbits maintained on a low salt diet did not increase fluid absorption or cell volume. We conclude from this that the DOCA-induced increases in fluid absorption and cell volume are not due to a direct mineralocorticoid action, but to indirect effects realted to factors such as increased extracellular fluid volume, increased glomerular filtration rate or potassium depletion. Electron micrographs of perfused tubules from DOCA-treated and normal rabbits revealed no consistent major morphologic differences.

PHS-6040

Objective

The objective of this project is to determine the site and mechanisms of hormonal control of renal sodium and potassium transport using primarily the *in vitro* perfused tubule technique developed in this laboratory.

Methods

Current focus is on *in vitro* tubule perfusion studies in two nephron segments:

1) *Proximal straight tubule*. Because fluid absorption is isosmotic in this segment, measurement of fluid absorption serves as an indicator of net sodium absorption. Intrinsic rates of fluid absorption are determined following different *in vivo* treatments of the rabbits.

2) *Cortical collecting duct*. Electrolyte transport in this segment is assessed either isotopically or by helium glow photometry. The *in vitro* effects of various peptide and steroid hormones on electrolyte transport will be studied with the goal of determining which hormones have physiologic actions on this segment and what mechanisms are involved in these actions.

Major Findings

Rates of fluid absorption by outer cortical proximal straight tubules from rabbits which have undergone various *in vivo* treatments are as follows:

Treatment	Number of tubules	Fluid Absorption ($nl \cdot mm^{-1} \cdot min^{-1}$)
Normal	17	0.60±0.06 (SE)
Chronic deoxycorticosterone (DOC) administration	12	1.00±0.04*
Low sodium diet	5	0.43±0.12
Low sodium diet & DOC	6	0.64±0.08

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*$p<0.01$ vs. normal

Rates of fluid absorption in outer cortical proximal straight tubules from adrenalectomized and sham-operated rabbits were not different from normal control values. Because neither very high circulating levels of endogenous aldosterone (low sodium diet) nor very low levels (adrenalectomy) were associated with changes in the intrinsic rate of fluid absorption, these studies do not provide evidence for a direct mineralocorticoid effect on sodium absorption by proximal straight tubules. The increase in fluid absorption in proximal straight tubules from DOC-treated animals is most likely an indirect effect of the hormone secondary to changes in extracellular fluid volume, potassium balance, or glomerular filtration rate.

DOC administration also resulted in an increase in cell height and cell volume per unit length in the perfused proximal straight tubule. However, there were no changes in cellular dimensions in tubules from rabbits treated with low sodium diet, low sodium diet plus DOC, or adrenalectomy. Thus, as with the DOC-induced increase in transport, the increase in cell size probably does not represent a direct mineralocorticoid action of DOC.

Significance

Although much has been learned in recent years about mechanisms of renal tubular epithelial transport of sodium and potassium, little is known about mechanisms of control of electrolyte transport in response to imposed changes in electrolyte and water balance. Aside from the obvious importance to basic renal physiology, knowledge of such control mechanisms could add measurably to the understanding of the pathophysiology of various human disease states such as essential hypertension.

Proposed Course

Attention is switched to the cortical collecting duct, an important site of control of sodium and potassium secretion. Because there are many important uncertainties about the mechanisms of action of adrenal steroid hormones and peptide hormones on electrolyte transport in the collecting duct, experiments will be done to assess the effects of these agents on electrolyte transport when added to the tubule *in vitro*. Depending on the results of these studies, further experiments will be carried out to determine the mechanisms involved in intracellular mediation of the hormonal signal and in alterations in transport.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01231-03 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Acid base regulation by cortical collecting ducts

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Jess Boyer, M.D., Guest Worker, KE/NHLBI

Other: Maurice B. Burg, M.D., Chief, KE/NHLBI
James Atkins, M.D., Ph.D., Research Fellow, KE/NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.3	0.2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to delineate the mechanism and control of acidification in the cortical collecting duct. Bicarbonate transport and pH are measured in isolated perfused collecting ducts dissected from rabbits. The role of various other ions in modulating bicarbonate transport is being tested.

PHS-6040
(Rev. 10-76)

Objective

Recent studies from our laboratory showed that bicarbonate transport in cortical collecting ducts perfused in vitro was affected by the acid-base status of the animals from which they were dissected; i.e. tubules from acidotic animals absorbed bicarbonate and tubules from alkalotic animals secreted bicarbonate. The purpose of the present work is to investigate factors that modulate bicarbonate absorption and secretion and pH in the cortical collecting duct.

It has been suggested, based on indirect evidence, that the salt retaining ability of an animal, and the type and concentration of anion in tubular fluid affect acid-base excretion. We began by looking at these possible modulators of collecting duct bicarbonate transport.

Methods

Cortical collecting ducts are dissected and perfused as previously described. Bicarbonate is measured by the technique of microcalorimetry developed in this laboratory. A micro pH electrode, also developed in this laboratory, is used for measuring pH in collected tubular fluids. Some of the animals were made acidotic with NH_4Cl and some were made alkalotic with $NaHCO_3$. Other animals were maintained on a low sodium diet.

Major Findings

1. Sulfate, a large anion present in normal urine, decreases bicarbonate secretion. It does not affect the bicarbonate absorptive process.

2. The animals maintained on a low sodium diet had increased salt retaining ability. Tubules removed from these animals, and perfused in vitro, had bicarbonate secretion which was blocked by sulfate.

3. A further miniaturization of the micro pH electrode allows for extremely slow perfusion rates without significant CO_2 loss. This will allow measurement of pH in the presence of the HCO_3^--CO_2 buffer system in any tubular segment.

Significance

The collecting duct can make the final adjustments in urinary pH. Factors affecting proton and bicarbonate transport in both the cortical and medullary collecting ducts presumably are major determinants of urinary acid-base excretion. The present studies indicate that sulfate, or anions like it, may modulate the process by inhibiting bicarbonate secretion in the cortical collecting ducts.

Proposed Course

1. Using the pH electrode, investigate the mechanism of acidification in the presence and absence of the HCO_3^-/CO_2 buffer system in the proximal tubule as well as the cortical collecting duct.

2. Examine hormonal influences on acidification in the cortical collecting duct.

3. Investigate both bicarbonate transport and pH in the medullary collecting duct.

4. Further explore techniques for independently evaluating proton secretion (bicarbonate absorption) and bicarbonate secretion.

Publications

1. Burg, M. B. and Iino, Y.: Control of renal bicarbonate transport. Satellite Symposium of Epithelial Transport Mechanism, Kyoto, Japan , Membr. Biochem. 2:405-411, 1979.

3. Burg, M. B.: Secretion and reabsorption of bicarbonate in single renal tubules of the rabbit, N. Y. Acad. Sci. (in press).

3. Burg, M. B. Bicarbonate transport by rabbit renal proximal tubules. University of Otago, Dunedin, New Zealand, 1980. (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01234-03 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Osmolality of the lateral intercellular spaces of a living epithelium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Kenneth R. Spring, DMD, Ph.D., LKEM/NHLBI

Other: Anna J. Coble, Ph.D., Guest Worker, LKEM/NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION
Electrolyte Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	1-1/2	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The osmolality of the fluid within lateral intercellular spaces separating gallbladder epithelial cells is being determined from measurements of the refractive index. Living Necturus gallbladder epithelium is placed in a special chamber and observed with a microscope interferometer. The refractive index of the cells and the spaces between them are being compared to a surrounding cell-free area.

Objective

Fluid transport by epithelia is secondary to active solute transport into the lateral intercellular spaces separating epithelia cells. Several recent theories predict that the spaces are filled with a fluid similar in concentration to that in the bulk solutions bathing the tissue, while the earlier models indicated that the intercellular fluid is more concentrated than the bulk solution. It is the object of this project to measure the osmolality of the intercellular fluid by determination of its refractive index, in situ, to resolve the question.

Methods

Necturus gallbladder is mounted in a special perfusion chamber after removal of the serosal connective tissue. This chamber permits the continuous perfusion of both mucosal and serosal baths and ideal optical properties. The tissue is supported on a titanium electron microscopy grid and is observed at 1000x magnification on a Mach-Zehnder microscope interferometer. Video images of interference fringe patterns are analyzed illumination at several different wave lengths.

Major Findings

We have been able to measure the osmolality of test solutions within a few percent. Similar accuracy has been achieved on the living epithelium. Difficulties in interpretation of the tissue measurements previously prevented meaningfull calculations of interspace osmolality. Interspace fluid osmolality has been determined in transporting and non-transporting states, and the refractive index computed by two methods. The results with the two-wavelengths method (narrow fringes at two or more wavelengths of monochromatic blue-green were corrected for interferences from the junctional complex and overlying cytoplasm.

Proposed Course

We are presently processing data from our experiments on Necturus gallbladder. We project completion of our data processing by approximately Sept. 1, 1980. The results will include junctional complex thickness and refractive index, and intercellular space refractive index and thickness. Interspace fluid osmolality may then be calculated from the refractive index value.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01237-02 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Hormonal control of transport in a line of toad kidney epithelial cells in culture

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. O.: F.M. Perkins, M.D., Guest Worker, LKEM/NHLBI

Other: J. S. Handler, M. D., Hd, Sec. on Membrane Metabolism LKEM/NHLBI
C. Watlington, M.D., Guest Worker, LKEM/NHLBI
A. S. Preston, Chemist, LKEM/NHLBI
R. Steele, Ph.D., TD/NHLBI
P. Munson, Ph. D., E&RR/NICHHD

COOPERATING UNITS (if any)
Lab. of Technical Development, NHLBI
Endocrinology & Reproduction Research Branch, NICHHD

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION
Membrane Metabolism

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS: 2.4	PROFESSIONAL: 2.4	OTHER: 0

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Toad kidney cells derived from Xenopus laevis form a monolayer epithelium of high electrical resistance. The cells transport sodium from the apical to the basal-lateral bathing solution generating a potential difference. The short-circuit current is equivalent to the net sodium transport. Sodium transport is stimulated by aldosterone. Aldosterone occupancy at specific nuclear binding sites correlates in a linear manner with the stimulation of sodium transport. The aldosterone effect is readily reversible. Incubation with aldosterone results in increased ouabain binding to membrane preparations. This stimulation is blocked by amiloride. Cyclic AMP analogues stimulate sodium transport.

PHS-6040
(Rev. 10-76)

Objective

Work in this laboratory and others has demonstrated that epithelia formed in culture have transport properties and responses to hormones and drugs that are like those of naturally occurring epithelia. The objective of this study is to analyze the properties of epithelia formed by cells of the line A-6, derived from the kidney of Xenopus laevis, and to explore the mechanism of transport and its control.

Methods

See last years report and the report of R. Steele, LTD, NHLBI.

A new and more efficient method for measuring nuclear binding of aldosterone was developed. The standard technique is to homogenize the cells and separate nuclei by centrifugation, as in last year's report. In the new method, the cells are made permeable by transfer to a cold, dilute buffer. After 10 min. the suspension is filtered through a millipore filter in a fashion that results in retention of aldosterone bound to nuclear sites. The method was validated by comparing results of the new method and the standard method after cells were incubated with a wide range of concentrations of aldosterone for long and short periods.

The number of active sodium transporters is assessed by the specific binding of ^{3}H-ouabain in whole homogenates of the epithelia. Assay conditions have been refined so that maximal specific binding is achieved (non-specific binding is less than 5% of total binding).

Major Findings

A-6 cells form a monolayer epithelium with a high transepithelial electrical resistance of 5000-8000 ohm-cm^2, mean potential difference of 10mV, apical surface negative, and mean short-circuit current (I_{SC}) of $1.0\mu A\cdot cm^{-2}$. I_{SC} is stimulated after 3-8 hours of incubation with aldosterone and after 10-15 minutes of incubation with 8-BrcAMP or other active analogs of cAMP. I_{SC} is equal to net sodium transport under all these conditions.

The initial steps in the action of aldosterone have been characterized by measuring nuclear binding of the hormone. The cells metabolize aldosterone to a more polar material which is not displaced by aldosterone from binding sites and is included in non-specific binding. Because of the metabolism, binding characteristics are regarded as approximations and are termed "apparent." There are two classes of binding sites, one with an apparent affinity $(K_D')=10^{-10}$M, which binds 15 fmol per mg protein, the other with $K_D'=10^{-8}$M which binds 160 fmol per mg protein. The two sites also differ in their relative affinity for a series of steroid hormones. Occupancy of the low affinity binding site correlates with stimulation of I_{SC} by aldosterone.

Because of our ability to study these epithelia for a long time, we have been able to follow the time course of the stimulation of I_{SC} by aldosterone and its reversal when aldosterone is withdrawn. Stimulation is evident after 90 min and reaches maximal I_{SC} after 8 hours. Reversal is complete within 24 hours.

Incubation with 10^{-7}M aldosterone, a concentration that stimulates I_{SC} maximally, results in more active sodium transporters (assayed by specific binding of ^{3}H-ouabain). The increase is evident as early as 6 hours after addition of aldosterone. Maximal increase occurs at 12-24 hours and is 40-60% greater than controls. In order to test whether the aldosterone-elicited increase in ouabain binding requires increased sodium transport, amiloride was added to the incubation (@aldosterone). Amiloride blocks 90-95% of sodium transport by these cells. Incubation with amiloride had no effect on the amount of ouabain bound by control cells, but markedly reduced the stimulation of binding by incubation with aldosterone. These results are interpreted as indicating that the increased ouabain binding by aldosterone treated cells is not directly induced by the hormone, but is secondary to the stimulation of sodium transport.

Based on the incorporation of radiolabelled amino acids into proteins, aldosterone stimulates the synthesis of proteins of 66,000 and 29,000 molecular weight (in SDS gels). The proteins are most evident in a microsomal fraction.

Significance

The study has clarified the relationship between nuclear binding of aldosterone and its effect on sodium transport. The preparation is ideal for further study of this early step in the action of this hormone. The increase in sodium transporters resulting from increased sodium transport following aldosterone has not been previously demonstrated so directly. It should lead to a better understanding of the control of this important transport enzyme.

Proposed Course

The regulation of nuclear binding sites for aldosterone will be studied by assessing the effect of other hormones and growth conditions on binding of aldosterone. The molecular weights of the proteins whose synthesis is stimulated by aldosterone is similar to the molecular weights of some of the subunits of Na-K-ATPase. The possibility that they are parts of the enzyme will be tested by antibody precipitation of the enzyme and by examining amino acid incorporation in the presence of amiloride which blocks the increase in the number of sodium pumps produced by aldosterone.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01240-01 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Na-K-ATPase in isolated segments of renal tubules

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Lal C. Garg, Ph.D., Guest Worker, KE/NHLBI

Other: Maurice B. Burg, M.D., Chief, KE/NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2/3	2/3	none

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Na-K-ATPase has been found to be different in various segments of rabbit nephron from 0 to 200 pmole/mm tubule length/minute associated with the difference in the rates of Na^+ reabsorption in various segments. Furthermore, Na-K-ATPase activity was found to be stimulated in cortical collecting ducts by pretreatment of rabbit with DOCA as in the case with Na reabsorption and K secretion.

PHS-6040
(Rev. 10-76)

Objective:

Because of the importance of the Na-K-ATPase in the renal reabsorption of Na$^+$, we wanted to develop a sensitive but simple method for assaying this enzyme in the isolated tubule segments. Furthermore, we wanted to study and compare the Na-K-ATPase activity of various tubular segments. Finally, we wanted to study the effect of mineralocorticoids (either by giving deoxy corticosterone acetate or keeping the animal on a low salt diet) on Na-K-ATPase in various segments of the rabbit renal tubules.

Methods:

The Na-K-activated ATPase was analyzed by the modification of the method of Schoner, et. al. (1967). The method couples ATP hydrolysis with oxidation of NADH as follows:

$$\text{ATP} \xrightarrow[\text{Na-ATPase}]{Mg^{++},\ Na^{+},\ NH_4^{+}} \text{ADP} + \text{Pi}$$

$$\text{Phosphoenolpyruvate} + \text{ADP} \xrightarrow[\text{Pyruvate Kinase}]{Mg^{+},\ NH_4^{+}} \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow[\text{Lactate Dehydrogenase}]{} \text{Lactate} + \text{NAD}$$

There is a Stoichiometric relationship between the oxidation of NADH and the hydrolysis of ATP.

We followed the rate of NADH oxidation fluorimetrically in a specially modified fluorometer (Czaczkes, et. al. 1979) which is more sensitive than the spectrophotomoetric measurements used by Schoner et al (1967).

Major Findings:

1. We developed a microassay for the determination of Na-K ATPase activity in the isolated segments of the nephron. The method is sensitive, reproducible and simple.

2. In normal rabbits, Na-K-ATPase activity was found to be very high (100-200 pmole/mm/min of ADP formation) in early proximal convoluted tubule (S1), medullary thick ascending limb, distal convoluted tubule and connecting tubule; low (15-40 pmole/mm/min) in proximal straight tubule (S2 and S3) cortical ascending limb and the collecting duct system and zero in the thin limbs.

3. Chronic treatment of rabbits with deoxycorticosterone acetate (5mg/day for 10-12 days) or low salt diet greatly increased Na-K-ATPase activity in cortical collecting duct without comparable changes in the other segments.

Significance:

The development of simple microassay for Na-K-ATPase activity will be useful in determination of the effect of physiological and pharmacological agents on this enzyme in various segments of the nephron which may help in elucidating the regulatory mechanism of renal Na^+ reabsorption.

Proposed Course: Project completed.

Publication: In preparation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01241-01 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Ionic composition of Necturus gallbladder cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Kenneth R. Spring, DMD, Ph.D., Staff Physiologist, KE/NHLBI

Other: Richard S. Fisher, Ph.D., Staff Fellow, KE/NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/2	1/2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Intracellular ionic activities of potassium and chloride are being measured in Necturus gallbladder epithelial cells undergoing osmotically induced volume changes.

PHS-6040
(Rev. 10-76)

Objectives

Necturus gallbladder epithelial cells undergo volume changes when exposed to solutions whose osmolality differs from control solutions. Osmotically induced cell shrinkage or swelling is followed by a rapid readjustment of cell volume back toward the control volume. Such readjustment is termed "volume regulation: and in other tissues results from the flow of solutes into or out of the cell. The object of these experiments is the identification and quantitation of the ion movements responsible for the observed volume regulation

Methods

Ion sensitive microelectrodes have been constructed. The electrodes are made from microfiber glass capillaries drawn to a tip of diameter less than 1 μm. The electrodes are siliconized by dipping the tip into trichloromethylsilane for a few seconds and then heating them. The electrodes are stored unfilled until just before use when they are filled from the back end with liquid ion exchange resin. The ion exchangers used in these experiments were sensitive to Cl^- or K^+. Since the ion sensitive electrode has a very high electrical resistance (10^{10} ohms) it exhibits slow response time and great sensitivity to electrical interferences. It is connected to a high impedance electrometer whose output is displayed on a digital voltmeter, interfaced to a small computer. Determination of the end point of electrode drift and slope sensitivity to K^+, or Cl^- is then made objectively by this computer system. A chamber has been designed which permits rapid exchange of the solutions bathing either surface of the gallbladder epithelium.

Major Findings

The experiments are in a very early stage and most of our efforts have been devoted toward the fabrication of working electrodes of suitable geometry. We have also evaluated the speed of exchange of solutions in our chamber and have modified the chamber design to reduce the time required for a complete change of solution.

Proposed Course

We will determine intracellular chloride activity and cell membrane potential during osmotically induced cell shrinkage and subsequent volume regulatory swelling. We will investigate the effects of ouabain and other inhibitors on intracellular composition and cell volume regulation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01242-01 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Electrophysiology related to ion transport in distal nephron segments

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: Antonio Almeida, Ph.D., Visiting Scientist, KE/NHLBI

Other: Maurice B. Burg, M. D., Chief, KE/NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION
Renal Mechanisms

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.9	.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The objective of the project is to measure transepithelial electrical resistance in the connecting tubule, in the distal convoluted tubule, in the medullary collecting tubule, and in the medullary thick ascending limb of Henle's loop.

PHS-6040
(Rev. 10-76)

Objective:

Following the development in this laboratory of methods to perfuse individual nephron segments in vitro, sodium and potassium transport in most of the twelve mammalian nephron segments has been exhaustively analyzed. Notable exceptions are distal convoluted tubules and connecting tubules. measurements in those segments are limited to adenylate cyclase, sodium, potassium-activated ATPase, osmotic water permeability, and transepithelial voltage. The characteristics of sodium and potassium transport have not been directly examined, despite the fact that the sodium, potassium-activated ATPase is high and this enzyme activity correlates with sodium transport in other segments. The reason that the distal convoluted and connecting tubules have not been more intensively studied is that they are difficult to identify and dissect, and in many nephrons are very short (<0.5 mm). The present project is directed towards these difficult but important, studies. The initial steps will be measure transepithelial voltage and resistance, since those measurements are relatively simple and can be performed on short tubule segments.

Methods:

The method previously developed and described from this laboratory were being used for tubule dissection and measurement of transepithelial voltage and resistance.

Major findings:

Dr. Almeida has been learning the methods by reconfirming previous findings with cortical collecting ducts. This accomplished, he is now dissecting and perfusing connecting tubules.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01243-01 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
D-glucose transport across the renal proximal tubule brush border membrane

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: R. James Turner, Ph.D., Visiting Associate, LKEM/NHLBI

Other: A. Moran, Ph.D., Visiting Associate, LKEM/NHLBI
M. Silverman, M. D., Dept. of Medicine, University of of Toronto, Toronto, Canada

COOPERATING UNITS (if any)
Dept. of Medicine, University of Toronto, Toronto, Canada

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
.9	.9	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The kinetics and specificity of D-glucose transport across the renal proximal tubule brush border membrane are being studied in vesicle preparations.

700

Objectives

The continuing goal of this project is to elucidate on both the physiological and molecular level the mechanism by which D-glucose is reabsorbed from the urine in the proximal tubule. At present we are concentrating on the specificity and kinetic properties of the transporter as studied in isolated brush border membrane vesicles.

Methods

Techniques for preparing purified proximal tubule brush border membrane vesicles have been worked out for both the dog and the rabbit. These are essentially modifications of previously published methods of our own and others. Purity is monitored by assaying for enzymes known to be localized to the brush border membrane, the antiluminal membrane, mitochondria, endoplasmic reticulum and lysosomes.

Transport and binding studies are carried out using radiolabelled substrates and rapid filtration techniques. A fast sampling apparatus capable of sampling times as short as 0.5 seconds has been constructed with the assistance of Dr. G. Vurek (Laboratory of Technical Development, NHLBI).

Major Findings

The specificity and kinetic characteristics of brush border membrane D-glucose transport have been studied in some detail by ourselves and others. Two significant contributions to the understanding of this process have come from our laboratory this year. The first arises from a study of the phlorizin binding properties of the transporter and the second deals with heterogeneity of transport sites along the length of the proximal tubule. Some complimentary theoretical studies have also been carried out.

(a) Phlorizin binding studies (with M. Silverman)

The compound phlorizin is a potent competitive inhibitor of renal D-glucose reabsorption. It acts by competing with the sugar for its binding site on the brush border transporter without itself being translocated. As a result of the less complex interaction of phlorizin with the carrier (binding for the inhibitor vs. binding, translocation and dissociation for substrate) it is possible to obtain certain types of information from phlorizin binding studies which are considerably more difficult to obtain from transport experiments. We have shown that the binding of sodium and phlorizin to the brush border glucose carrier occurs via a random rather than an ordered process and that the stoichiometry of these binding events is one sodium ion per phlorizin molecule. Binding constants and other kinetic parameters for the interaction of phlorizin and sodium with their receptors have also been determined. These results suggest that the binding of glucose and sodium to the transporter is a random process and thus that models of the transporter which require ordered binding (e.g. certain types of pores) are incorrect.

(b) Glucose transport studies

Early experiments by ourselves on the kinetics of D-glucose transport in brush border vesicles indicated that more than one transport site might be present in these preparations. Our suggestion at that time was that these results might indicate a heterogeneity of transporters along the length of the proximal tubule. We have now succeeded in physically separating two distinct sodium dependent, phlorizin sensitive glucose transporters from rabbit kidney. The first, a low affinity high capacity system, is seen in vesicle preparations from the outer cortex containing predominately brush border membranes from the earliest part of the proximal tubule. The second, a high affinity low capacity system, is seen in preparations from the outer medulla presumably containing predominately membranes from the last part of the proximal tubule. Preliminary experiments also indicate differences in sodium dependence and in specificity for glucose analogues between these two transport sites.

(c) Models of coupled transport systems

In order to better understand the possible mechanisms of coupled transport and to design appropriate experiments to distinguish between them, we have been analyzing the properties of a family of co-transport models. Although these models are based on the carrier principle they are not limited to the traditional mobile carrier interpretation. The most general model allows the substrate molecule and its co-transported cation to bind in a random fashion at either membrane face. Various submodels include ordered binding schemes and pore-type models where the order of binding is reversed at the two membrane faces. We have shown that the various transport models can be distinguished from one another on the basis of a series of relatively simple kinetic measurements. Rejection criteria for carrier-type models in general have also been derived.

Proposed Course

A detailed study of the glucose transport and phlorizin binding properties of the high affinity and low affinity glucose transporters is planned.

Publications

1. R. James Turner and M. Silverman. Sugar uptake into brush border vesicles from normal human kidney. Proc. Natl. Acad. Sci. 74:2825-2829, 1977.

2. R. James Turner and M. Silverman. Sugar uptake into brush border vesicles from dog kidney. I. Specificity. Biochim. Biophys. Acta 507:305-321, 1978.

3. R. James Turner and M. Silverman. Sugar uptake into brush border vesicles from dog kidney. II. Kinetics. Biochim. Biophys. Acta 511:470, 1978.

4. R. James Turner and M. Silverman. Testing carrier models of co-transport using the binding kinetics of non-transported competitive inhibitors. Biochim. Biophys. Acta 596:272, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01244-01 KE

PERIOD COVERED
October 1, 1980 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Vesicle preparations from cultured epithelial cell lines

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I.: R. James Turner, Ph.D., Visiting Associate, LKEM/NHLBI

Other: J. Handler, M. D., Hd. Sec. on Membrane Metabolism, LKEM/NHLBI
F. Perkins, M. D., Guest Worker, LKEM/NHBLI
A. Moran, Ph. D., Visiting Scientist, LKEM/NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are developing vesicle preparation procedures for several cultured cell lines. Functional membrane vesicles have been obtained from the A6 (toad kidney) and LLC-PK_1 (pig kidney) cell types.

704

Objectives

Our long term objective is to combine the advantages of studying transport processes in intact cultured epithelia with those inherent to an isolated vesicle preparation. We plan to follow the effects of various drugs and hormones on selected epithelial transport systems. The production of the desired biological effect will be monitored using intact cells, then the transport-related events will be studied in the vesicle systems. An important part of this work concerns the development of techniques for isolating and distinguishing between apical and basal plasma membranes.

Methods and Major Findings

We have explored a number of methods for homogenizing cells (hypotonic shock; nitrogen cavitation; Polytron, Potter-Elvehjen, Dounce and VirTis homogenization; and combinations of the above) and for isolating plasma membranes (differential centrifugation; density gradient centrifugation and calcium precipitation). A method based on a combination of hypotonic shock, VirTis homogenization and calcium precipitation seems to produce a good yield of plasma membranes for those cell types tested. A series of enzymatic and other markers has been developed to monitor the purity of the final vesicle fraction. These are alkaline phosphatase (some apical membranes), Na-K-ATPase (basal-lateral membranes), glucose-6-phosphatase (endoplasmic reticulum), acid phosphatase (lysosomes), succinic dehydrogenase (mitochondria), 5-nucleotidase (plasma membranes) and DNA (nuclei). Preliminary attempts to label the apical membranes of A6 cells with ^{3}H-DIDS have also been carried out.

To date plasma membrane vesicles have been prepared from the A6 line derived from toad kidney and the LLC-PK$_1$ line derived from pig kidney. Preliminary experiments on A6 vesicles indicate a saturable amiloride sensitive component of sodium transport. Vesicles prepared from the LLC-PK$_1$ line show sodium dependent, phlorizin sensitive D-glucose transport.

Proposed Course

A detailed study of the glucose and phosphate transport properties of LLC-PK$_1$ vesicles is planned for the future.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 HL 01245-01 KE

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Amiloride inhibition of a volume-stimulated Na/H antiport (or Na/OH symport) in Amphiuma red cells

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P. I. Floyd M. Kregenow, M.D., Sr. Invest., LKEM/NHLBI

Other: Theresa Caryk, Chemist, LKEM/NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Kidney & Electrolyte Metabolism

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, Bethesda, MD

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/2	8/12	10/12

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Amphiuma red cells enlarge and regulate their volume by utilizing in amiloride-sensitive Na transport mechanism. Cl is not essential to this transport process unlike the comparable response in duck erythrocytes. The Amphiuma transport process acts as a Na/H antiport or alternatively as a Na/OH symport. As such it is capable of transferring the energy inherent in a cation (Na) electrochemical gradient maintained across a membrane to an electrochemical gradient involving H ions.

706

PHS-6040
(Rev. 10-76)

Objectives

Some animal cells can regulate their volume by altering their salt content. They use ouabain-insensitive transport mechanism(s) to either add to or reduce their ion content. Osmotic shifts in cell water follow and produce the necessary changes in cell size. These transport mechanisms constitute part of a "volume controlling mechanism" which has been studied most extensively in the nucleated erythrocytes from two animals, duck and Amphiuma.

Recently, the results, using three experimental approaches have increased our undertsanding of the transport mechanism(s) in duck red cells. We measured in these three experimental approaches: a) extracellular pH when transport and volume regulation takes place in a minimally buffered media, b) Na, K and Cl transport after having substituted Br,I, SCN, or NO_3 for Cl or blocked the anion exchange mechanism of red cells with the disulfonic stilbene, SITS, and c) the membrane potential using the carbocyamine dye, $DISC_3$.

These studies indicate that the basic transport process(es) in duck red cells involves electroneutral salt translocation in which equivalent amounts of Cl accompany both Na and K. As duck erythrocytes enlarge, for instance, they utilize a Na plus K plus Cl co-transport system to acquire additional Na, K and Cl. This transport process shares several similarities with the furosemide-sensitive transporter(s) of epithelial cells.

During the past year we have applied these same three experimental approaches to the transport process responsible for enlarging Amphiuma red cells in an effort to better understand this transport system. These results indicate that unlike duck erythrocytes, the basic transport process in Amphiuma red cells acts as an antiporter which exchanges Na for H in a one to one fashion. Alternatively this process can be viewed as a symporter which co-transports Na and OH.

Our interest in the Amphiuma transport process is an outgrowth of previous studies and has rather general interest. We reported previously (Siebens & Kregenow, Annual Reports, 1978, 1979) that the Amphiuma transport process is blocked by amiloride and shares several characteristics with the amiloride-sensitive transporter of epithelial cells. Thus there exists the possibility that the furosemide and amiloride sensitive transport systems responsible for epithelial electrolyte transport may be similar or identical to those that regulate cell volume in duck and Amphiuma erythrocytes.

Methods

The basic techniques and procedures have been described in previous annual reports.

Major Findings

If Amphiuma erythrocytes are allowed to enlarge in a hypertonic solution which has very little buffering capacity, the medium becomes acidic. This acidification process requires that cells enlarge; amiloride by inhibiting Na uptake prevents enlargement and almost completely inhibits acidification. Acidification is an integral part of the enlargement process, corollating with both Na uptake and the increase in cell size. Changes in medium cation and anion composition, osmolality and pH which produce very characteristic changes in the rate of Na uptake produce identical changes in the rate of acidification.

This acidification process is actually a buffered response although measurements are obtained in a minimally-buffered media. The anion exchange mechanism of red cells, by permitting Cl and HCO_3 to exchange across the membrane, allows extracellular H or OH, in effect, to react with the cellular buffering system. One can obtain direct evidence in support of this explanation for pH equilibration across the membrane by following the shifts in medium pH after introducing a bolus of NaOH to the medium when the medium is rendered low in HCO_3 or contains anions other than Cl. This sort of analysis also demonstrates that treating cells with SITS, which blocks the anion exchanger, prevents any pH equilibration from taking place across the membrane at all.

The medium becomes acid because as cells enlarge there is a disparity in the number of new cations and anions that eventually end up on the opposite side of the membrane. More Na than Cl is removed from the medium. Thus, hydronium and hydroxyl ions become important in maintaining electroneutrality on both sides of the membrane. Measurements of the amount of titrable acidity that develop during the response support this explanation. Titrable acidity was measured in a fairly well-buffered medium and calculated from the amount of NaOH needed to maintain medium pH constant. After a thirty-minute incubation, titrable acidity is approximately 45% of the amount of Na taken up when both are expressed in millimoles and agrees with the 40-50% predicted from the differences in the net uptake of Na and Cl under similar conditions.

Anion substitution experiments applied to the Amphiuma transport process demonstrate that Cl is not essential to the Amphiuma response, unlike in the duck response. Br again substitutes for Cl but so can NO_3. I and SCN replacement cause only partial inhibition of Na uptake. One can demonstrate more directly that Cl is not essential to the Na uptake process of Amphiuma red cells by studying SITS-treated cells. SITS-treated cells still take up large quantities of Na but no Cl, and they show no amiloride-sensitive change in Cl fluxes.

Treating Amphiuma red cells with SITS, also augments the medium acidification, described previously, that takes place when the transport system is activated. Part of this augmented pH change results from SITS having blocked the anion exchanger and impaired the cell's ability to pH regulate. The medium becomes more acid because extracellular hydrogen ions can no longer interact indirectly with the cellular buffering system. But not all of the enhanced pH change is explicible in these terms. This becomes apparent if SITS-treated cells are permitted to enlarge in a fairly well-buffered medium and the titrable acidity measured as before. Under these conditions, Na uptake and the measured titrable acidity are approximately equivalent. There is an apparent one to one exchange of Na for hydrogen at all Na concentrations.

The following model is consistent with the data. Na enters the cell through an amiloride-sensitive Na/H antiport or Na/OH symport. By blocking the anion exchanger and associated Cl pathway, SITS prevents the normal passive distribution of chloride and hydroxyl. As Na enters SITS-treated cells, Cl is trapped outside while hydroxyl is trapped inside. The SITS-treated cell swells because osmotically active Na enters in exchange for hydrogen, which is not osmotically active when buffered by the cellular elements.

A finding consistent with the model is the observation that the membrane potential does not change appreciably during transport despite the large increase in Na uptake. The membrane potential was measured directly with microelectrodes using a single cell technique developed previously by this laboratory and indirectly using the carbocyamine dye, $DISC_3$. Both measurements are subject to criticism but taken together suggest an electrically silent Na uptake as proposed by the model.

The model also makes several predictions about the response of SITS-treated cells. Three of these were confirmed experimentally. First, Cl should not and does not enter as SITS-treated cells take up Na. Second, trapping hydroxyl ions within the cell should increase intracellular pH. Measurements of cell pH on frozen and thawed cells show that the intracellular pH does rise. Finally, one should expect a reduced cell volume per unit gain of cations with SITS-treatment since Na is no longer accompanied by osmotically active Cl. SITS-treated cells do show a smaller volume gain per unit gain in cation content.

Significance

1) The observations lead to a clearer understanding of transport process responsible for enlarging Amphiuma red cells. The findings are compatible with a model which depicts the transport process as a Na/H antiport or a Na/OH symport.

2) This transport process permits the cell to transfer energy from a cation (Na) electrochemical gradient to an electrochemical gradient involving H ions. This realization permits one to synthesize models for all duck and Amphiuma transport processes by making use of the chemiosmotic hypothesis of Mitchell.

3) The studies suggest that factors controlling intracellular pH may be important in certain stages of the volume regulatory responses of nucleated erythrocytes.

Publications

1. L. C. Stoner, F. M. Kregenow. A single cell technique for the measurement of membrane potential, membrane conductance and the efflux of rapidly penetrating solutes in Amphiuma red cells. J. Gen. Physiol. (In press for publication in October 1980).

2. F. M. Kregenow. Osmoregulatory salt transporting systems: Control of cell volume in anisotonic media. Ann. Rev. Physiol. (In press for Oct.-Nov. 1980).

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1979 THROUGH SEPTEMBER 30, 1980

The overall objective of the research program of the Molecular Disease Branch is the delineation of the molecular and structural properties of the human plasma apolipoproteins, the physiological role of apolipoproteins and lipoproteins in lipid transport, the determination of the mechanisms involved in the regulation of cellular cholesterol metabolism and transport, and the elucidation of the metabolic and molecular mechanisms involved in plasma lipoprotein biosynthesis, transport, and catabolism in normal individuals and patients with disorders of lipid metabolism and atherosclerosis.

During the last several years the staff of the Molecular Disease Branch has developed a conceptual framework for the understanding of the dynamic processes involved in the biosynthesis, transport, and catabolism of plasma apolipoproteins. Within this framework the plasma lipoproteins are conceptualized as a polydisperse collection of lipoprotein particles, the lipoprotein composition of which is determined by the laws of mass action. The important molecular constitutent of the plasma lipoprotein particle which is involved in the regulation of lipoprotein particle transport and metabolism is the apolipoprotein moeity. The distribution of a specific apolipoprotein within plasma is governed by the relative concentration of and affinity for, the plasma lipoproteins. This concept of plasma lipoproteins emphasizes the fundamental importance of the apolipoprotein in regulating lipoprotein metabolism and provides a model for understanding apolipoprotein-lipoprotein interactions during lipoprotein biosynthesis, transport, and catabolism in normal subjects and in patients with dyslipoproteinemia and atherosclerosis.

Prerequisite to an analysis of the physiological role of plasma apolipoproteins in lipid metabolism is a detailed knowledge of the biochemistry and molecular structure of the plasma apolipoproteins. Over the last several years we have undertaken a systematic study of the human plasma apolipoproteins including the primary structure of apoA-I, apoA-II, apoC-I, and apoC-III. Recently these studies have been extended to an analysis of apoD. ApoD has been isolated in homogeneous form by affinity chromatographic techniques. A larger molecular form of apoD was identified during apoD purification. Detailed characterization of this apolipoprotein revealed an apoD-A-II dimer. This disulfide dimer of apoD and monomeric apoA-II was present in most samples of high density lipoproteins (HDL) analyzed. Mixed dimers of apoA-II appear to be frequent within HDL, and have now been reported with apoE and apoA-I_{Milano}. The physiological importance of these mixed dimers is as yet unknown.

Studies during the last year have continued to focus on apoE. ApoE has been isolated in electrophoretically homogeneous form from normal subjects and patients with phenotypes III and V by affinity chromatography. Isoelectric focusing of apoE from normal and Type V subjects contained three principal isomorphic forms of apoE designated $apoE_1$, $apoE_2$, and $apoE_3$. All patients with Type III phenotype on isoelectric focusing were deficient in $apoE_3$. The amino acid compositions of apoE isolated from normal, Type III, and Type V

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subjects were similar. Two dimensional fingerprint analysis of apoE from normal and Type III subjects revealed that the Type III apoE appeared altered with the entire apolipoprotein shifted in pI. Detailed metabolic studies have been performed on apoE from normal, Type III and Type V subjects and will be discussed below.

Extensive investigation has also focused on a newly recognized apolipoprotein designated apoH. ApoH has been shown to be a major apolipoprotein constituent of lymph chylomicrons, and plasma chylomicrons-VLDL in patients with hypertriglyceridemia. This apolipoprotein has been shown to bind to artificial lipid emulsions, and to have a high affinity for plasma lipoproteins. Detailed characterization of apoH indicated that it was identical to β_2 glycoprotein-I which had been described several years ago as a glycoprotein component of plasma. Of major importance was the discovery that apoH activates lipoprotein lipase (see below).

Of particular interest over the last year has been the critical evaluation of the B apolipoprotein. Studies in the literature have suggested that in the rat apoB is present in two forms. The larger molecular weight form on SDS gel electrophoresis is the major protein constituent of LDL and is thought to be of liver origin. The smaller molecular weight form is the major apolipoprotein of lymph chylomicrons and has been proposed to be synthesized by the intestine. Studies in our laboratory have identified a large and small molecular weight B apolipoprotein which may be analogous to the apolipoproteins reported in the rat. These two apoB proteins have been purified and antibodies prepared against the individual forms. Detailed analysis of the two apolipoproteins are underway with respect to amino acid composition, molecular properties, and antibody specificity. These studies will be of particular interest since these two apolipoproteins may serve as biochemical markers for lipoproteins synthesized by the liver and intestine.

Of major importance in our characterization and analysis of plasma apolipoproteins has been the determination of the amino acid sequence of apolipoproteins. Studies on improving the methods for sequence analysis of peptides and proteins has continued in the Branch. A large reaction cup and cold trap have been developed for the commercial amino acid Sequencer. The large reaction cup increased the surface area of the cup by 75 percent. The large reaction cup and cold trap modifications have significantly improved the repetitive yield and decreased the nonspecific cleavage of proteins and peptides degraded on the modified Sequencer. These modifications have become available as accessory units for the commercial Sequencer, and should markedly facilitate the structural analysis of proteins and peptides.

A systematic analysis of the molecular properties of the human apolipoproteins continues to be an active area of research within the Branch. A detailed knowledge of the physico-chemical properties of the apolipoproteins is required for our understanding of the molecular mechanisms involved in apolipoprotein-lipoprotein particle interaction and metabolism. Within the framework of our concept of lipoproteins the quaternary structure of plasma lipoproteins and the changes in apolipoprotein composition of plasma lipoproteins during transport and metabolism is dependent on the molecular properties

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of the individual apolipoproteins. Studies within our Branch over the last several years have revealed that the human apolipoproteins have several unique molecular properties. Apolipoproteins A-I, A-II, and apoC-I have been shown to self-associate. The self-association scheme for the individual apolipoproteins is as follows: apoA-I, monomer-dimer-tetramer-octamer; apoA-II, monomer-dimer; apoC-I, monomer-dimer-tetramer. Of particular note was the recognition that concomitant with self-association of the apolipoprotein was a major increase in molecular structure. In the monomer form, apolipoproteins were nearly devoid of organized structure, whereas in the oligomeric form the conformation increased to that characteristic of globular proteins. These dramatic changes in structure are greater than those reported for any other self-associating protein system. The driving forces for the dramatic change in conformation of the apolipoproteins is the shielding of nonpolar residues from the solvent and is thus hydrophobic in nature.

The self-association of plasma apolipoproteins has been shown to be extremely sensitive to various ligands, pH, ionic strength, and protein concentration. Of particular note has been the demonstration of hydrostatic pressure on the molecular properties of the apolipoproteins. In interacting systems when there is a major change in solvent exposure of the nonpolar residues during oligomer formation there may be a significant change in molar volume. This is primarily due to the unfavorable interaction between nonpolar solutes and water. Presumably decreases in the amount of "ordered water" allow nonpolar solutes to expand when shielded from solvent. The association of apo-AI, apoA-II as well as apoC-I have now been shown to be significantly effected by pressure and the volume changes on associations are of a magnitude greater than previously reported in the literature.

During the past year the analysis of the molecular properties of apolipoproteins has been extended to apoC-III. In contrast to apoA-I, apoA-II, and apoC-I, there is little change in the structure of apoC-III with self-association. The secondary structure of apoC-III however does dramatically increase in the presence of inorganic phosphate. This interesting result illustrates the unique physico-chemical properties of the apolipoproteins.

Protein-protein interactions may be of major importance in the quaternary structure of plasma lipoprotein particles. The self-association of several of the apolipoproteins has been delineated as outlined above. Of equal importance were the nature and extent of mixed apolipoprotein interactions. The interactions of apoA-I + apoA-II, and apoA-II + apoC-I are currently being investigated. A detailed study of these mixed interactions has been facilitated by modification of one of the apolipoproteins under investigation by nitration, or sulfenylation. The molecular properties of these modified apolipoproteins were shown to be identical to the native protein. Employing these techniques we demonstrated the formation of specific mixed oligomers as follows: apoA-I:apoA-II, 1:1 and 2:2; apo A-II:apoC-I, 2:4.

The elucidation of the specific interactions between apolipoproteins will be of major importance as we continue to determine the role of apolipoproteins in the quaternary structure, function and metabolism of the plasma lipoprotein particles.

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During the last several years the cellular binding, uptake, and catabolism of plasma lipoproteins have attracted a great deal of interest and research. Investigation of the role of cholesterol in the atherogenic process has focused primarily on the etiological importance of plasma lipoproteins and cellular receptor sites. Of increasing interest and importance is the intracellular regulation of cholesterol esterification, hydrolysis, and biosynthesis. A key enzyme in the overall regulation of cellular cholesterol concentration is acid cholesteryl ester hydrolase, the lysosomal enzyme responsible for hydrolysis of cholesterol esters into cholesterol and free fatty acids.

Acid cholesteryl ester hydrolase (ACEH) activity has been assayed in mononuclear cells of patients (age 25-45) with angiographically documented coronary artery disease (CAD) and patients of similar age with structural heart disease. All established risk factors were evaluated in these patients. ACEH activity was significantly lower ($p<.005$) in patients with premature cardiovascular disease. Two established risk factors, smoking and low HDL-cholesterol were also statistically different between the control and CAD groups. However, multivariant analysis revealed the reduction in activity of ACEH in the CAD groups to be significant ($p<.005$) and independent of the incidence of HDL-cholesterol and smoking. In order to confirm the presence of low ACEH activity, skin fibroblasts were obtained from several patients with low mononuclear cell ACEH activity. Assay of ACEH activity in skin fibroblasts was also reduced to approximately one half the activity of ACEH activity in control cells. These results confirmed the enzyme defect in selected patients with premature CVD. A reduction in ACEH activity would be anticipated to play an important role in the atherogenic process by reducing cholesterol ester hydrolysis and decreasing the formation of free cholesterol subsequently available for removal from the cells. Based on these results, we have proposed that a deficiency in lysosomal ACEH activity may represent a new, independent risk factor for the development of premature cardiovascular disease.

One of the other major enzymes involved in intracellular cholesterol metabolism is HMG-CoA reductse, the rate limiting enzyme in the pathway for cholesterol biosynthesis. During the last several years the factors involved in the modulation of the enzymic activity of HMG-CoA reductase has been extensively studied in the Branch. HMG-CoA reductase has been isolatd in electrophoretically homogeneous form from chicken and rat liver. HMG-CoA reductase was shown to be present in enzymically active and inactive forms. Detailed studies established that the reversible inactivation of HMG-CoA reductase was due to covalent modification of the enzyme by a reversible phosphorylation-dephosphorylation reaction sequence.

The enzyme, reductase kinase, which catalyzes the phosphorylation of HMG-CoA reductase has also been purified to homogeneity. Reductase kinase, like HMG-CoA reductase, was shown to undergo reversible activation-inactivation. Studies employing a partially purified cytosolic protein kinase designated reductase kinase kinase, phosphoprotein phosphatase, and [^{32}P]-ATP have established that reductase kinase undergoes reversible phosphorylation-dephosphorylation. These results provide evidence that the *in vitro* enzymic activity of both HMG-CoA reductase and reductase kinase are modulated by a

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phosphorylation-dephosphorylation reaction sequence. The active form of HMG-CoA reductase is dephosphorylated, whereas the phosphorylated form of reductase kinase is active.

The availability of homogeneous HMG-CoA reductase and reductase kinase permitted a detailed analysis of the in vitro phosphorylation scheme. Incubation of purified HMG-CoA reductase with purified reductase kinase and [^{32}P]-ATP revealed a time dependent loss of enzymic activity and phosphorylation of HMG-CoA reductase. Analysis of radiolabeled HMG-CoA reductase revealed an average of approximately 4 mol of phosphate incorporated per mol of tetrameter of 200,000 molecular weight.

These results established that purified HMG-CoA reductase can be phosphorylated in vitro by purified reductase kinase. These studies are interpreted to indicate that the enzymic activity of HMG-CoA reductase is regulated by a bicyclic cascade system.

The regulation of the enzymic activity of HMG-CoA reductase has been extended to an analysis of the activity in vivo. Rats were injected with [^{32}P] and hepatic HMG-CoA reductase purified by immunoprecipitation with a monospecific antibody to HMG-CoA reductase or to homogeneity by affinity chromatography on HMG-CoA. Detailed study of the purified HMG-CoA reductase indicated that the enzyme undergoes phosphorylation in vivo.

Modulation of the in vivo phosphorylation of HMG-CoA reductase was analyzed following glucagon administration. Rats were injected with [^{32}P] and glucagon or saline, and HMG-CoA reductase isolated by immunoprecipitation or affinity chromatography as outlined above. The administration of glucagon was associated with a 40 percent decrease in enzymic activity of HMG-CoA reductase, a 10 fold increase in hepatic cyclic AMP content, a 2 fold increase in [^{32}P] incorporation into purified HMG-CoA reductase, and no change in hepatic ATP specific activity. These studies are interpreted to indicate that HMG-CoA reductase undergoes phosphorylation in vivo, and the degree of phosphorylation of HMG-CoA reductase may be modulated by polypeptide hormones. These combined results establish that the phosphorylation-dephosphorylation reaction sequence of HMG-CoA reductase is an important short-term mechanism for the modulation of cellular cholesterol biosynthesis.

The synthesis, transport, and catabolism of plasma lipoproteins continues to be an active area of investigation within the Branch. The role of apolipoproteins in the metabolism of plasma lipoproteins continues to be a focal point of study since the apolipoprotein is the unique constituent of the lipoprotein particle. The physiological function of several apolipoproteins has now been established. Apolipoproteins are known to serve as cofactors for enzymes (apoC-II and lipoprotein lipase, apoA-I and lecithin cholesterol acyltransferase), ligands for receptor binding (apoB and apoE), and exchange proteins (apoD-cholesterol ester exchange). Of particular interest are recent studies on the newly recognized apolipoprotein constituent of triglyceride rich lipoproteins, apoH (β_2-glycoprotein-I). ApoH has been isolated in homogeneous form as outlined above, and shown to be a constituent of chylomicrons and VLDL. ApoH was shown to activate lipoprotein lipase (LPL), the major enzyme involved in the hydrolysis of triglycerides of chylomicrons and

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VLDL. ApoH increased LPL activity by 45 +/- 17 percent in the presence of apoC-II. ApoC-III decreased the apoH+apoC-II enhanced LPL activity. These results provide evidence that the new apolipoprotein, apoH, modulates the enzymic activity of LPL in triglyceride metabolism. The molar ratios of apoH, apoC-II and apoC-III on triglyceride rich lipoprotein particles may influence the ultimate rate of LPL catalyzed triglyceride hydrolysis. Further studies on the role of apoH on triglyceride metabolism are currently underway.

Quantification of apolipoproteins represents a major prerequisite to the analysis of apolipoprotein concentration and metabolism in normal subjects and patients with dyslipoproteinemia. Immunochemical assays employing radial immunodiffusion or rocket electrophoresis have been developed and tested for several of the apolipoproteins including apoA-I, apoA-II, apoC-II, apoC-III, and apoB. Normal ranges and distribution within plasma density fractions have been ascertained. Quantification of apolipoproteins in normal individuals and patients with disorders of lipid metabolism and atherosclerosis are presently in progress.

The synthesis, transport, and catabolism of plasma lipoproteins continue to be an active area of investigation within the Branch. The site of synthesis of apolipoproteins has become of particular interest, and focus of research has shifted from the liver to also include the intestine. The intestine has now been recognized as a major site of apolipoprotein biosynthesis. We have adopted a new technique employing the immunoperoxidase procedure to analyze intracellular apolipoprotein biosynthesis. Utilizing this procedure we demonstrated that normal human jejunal tissue contains apolipoproteins A-I, A-II, and B. All three apolipoproteins were shown to be present in intestinal epithelial cells with the maximum staining present at the apical region of the villous. Intestinal biopsies from patients with Tangiers disease were analyzed and shown to contain apoA-I, apoA-II, and apoB. These results were consistent with data from metabolic studies which indicated that patients with Tangier disease were able to synthesize apoA-I, apoA-II, and B. The defect in this syndrome is not due to defective synthesis of apolipoproteins A-I and A-II, but rather due to accelerated apoA-I and apoA-II catabolism.

The role of the intestine in lipoprotein synthesis and transport has been further evaluated by analysis of the lipid and apolipoprotein transport in thoracic duct lymph. Transport of lymph triglyceride was approximately 83 percent of the daily ingested fat load while lymph cholesterol transport was consistently greater than the quantity of daily ingested cholesterol. Lymph apolipoprotein transport exceeded the predicted apolipoprotein synthesis rate of both apoA-I and apoA-II. The major quantity of lymph apoA-I and apoA-II was present within HDL. Lymph HDL particles are predominantly HDL_{2b}, and HDL_{2a}. They also have a greater triglyceride and reduced cholesterol ester content when compared to homologous plasma HDL. These combined results are consistent with a major contribution of the intestine to total body synthesis of apoA-I and apoA-II. An important role of lymph in recirculation of plasma apolipoproteins and lipoproteins is also suggested by these studies.

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The metabolism of apoA-I and apoA-II has been of particular interest recently due to the well established epidemiological data suggesting that HDL-cholesterol is a negative risk factor for the development of premature cardiovascular disease. Our laboratory has been investigating the metabolism of apoA-I and apoA-II for several years. Results from these studies have indicated that apoA-I and apoA-II synthesis rates in normal females are higher than in their male counterparts, while fractional catabolic rates are similar. This increased synthesis appears to account for the higher plasma apoA-I and apoA-II concentrations in females.

Plasma apoA-I and apoA-II are located principally within HDL. The density fraction of HDL has been further subdivided into HDL_{2b} (1.063-1.010 g/ml), HDL_{2a} (1.010-1.0125 g/ml), and HDL_3 (1.125-1.21 g/ml). Kinetic analysis of plasma apoA-I and apoA-II have now been interpreted to show that apoA-I is cleared from plasma more rapidly than apoA-II. The factors involved in the regulation of the concentration of the individual subfractions of HDL have been investigated by infusion of normal HDL into patients with Tangier disease who are deficient in plasma HDL. The half-lives for HDL_{2b}, HDL_{2a} were similar and significantly shorter than HDL_3. These data were interpreted to indicate that the metabolism of HDL_{2b} and HDL_{2a} was more rapid than HDL_3, and that plasma apoA-I is cleared from plasma more rapidly than apoA-II.

The concentrations of plasma HDL, and apolipoproteins A-I and apoA-II were decreased in patients with phenotypes I and V and abetalipoproteinemia. Detailed kinetic studies employ radiolabeled apoA-I and apoA-II have shown an increased rate of catabolism with no significant reduction in synthesis rates. Thus major changes in catabolism rather than synthesis account for the reduced plasma concentration of HDL. Based on the accumulated data from radiolabeled studies in normals and patients with dyslipoproteinemia a detailed kinetic model is being developed on HDL, apoA-I, and apoA-II metabolism.

The relative importance of HDL-cholesterol as a risk factor for the development of premature cardiovascular disease (CVD) has prompted a detailed review of the clinical course of patients with Tangier disease. The clinical features and CVD of all reported Tangier homozygotes (N=27) and Tangier heterozygotes (N=22) were reviewed. No CVD was present in subjects under 35 years of age. In subjects between 35 and 65 years of age, 5 of 19 heterozygotes (26%) and 5 of 11 homozygotes (45%) had some evidence of CVD as compared to 4% or less in a normal population. The data are consistent with the concept that patients with Tangier disease may be at increased risk for CVD, however that risk is not nearly as striking as that seen in homozygous familial hypercholesterolemia.

Metabolic studies on the E apolipoprotein have continued to be pursued in the Branch. ApoE is of major importance in the field of lipoprotein metabolism because it has been shown to bind to the LDL receptor, to be elevated in animals with experimental atherosclerosis and patients with Type III hyperlipoproteinemia. ApoE may also play a pivotal role in the uptake of lipoprotein remnants by the liver. ApoE of patients with Type III hyperlipidemia has been extensively studied and shown to be deficient in a polymorphic form of apoE, designated $apoE_3$, on isoelectric focusing. To further elucidate the importance of apoE in normal lipoprotein metabolism and in patients with

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Type III hyperlipoproteinemia we have initiated studies on the metabolism of normal apoE (designated $apoE_3^+$) and apoE from Type III subjects (designated $apoE_3^-$).

$ApoE^+$ was rapidly metabolized by normal subjects and represents the fastest catabolism of any human apolipoprotein studied thus far. $ApoE_3^+$ was catabolized faster ($p<.05$) than $apoE_3^-$ in normal and Type III subjects. These studies establish for the first time an altered catabolism of a human apolipoprotein in man. These results have been interpreted to indicate that the $apoE_3^-$ protein, characteristic of patients with Type III phenotype, is abnormal and metabolized at a significantly slower rate than $apoE_3^+$ isolated from normal subjects. This reduced catabolism is thought to be responsible for the delayed catabolism of lipoprotein remnants and the accumulation of β migrating LDL lipoproteins observed in Type III subjects. The abnormality of the $apoE_3^-$ protein is as yet unknown but may be related to a structural defect in the protein or a defect in post-translational modification of $apoE_3$ with failure to synthesize $apoE_3$. These results, which represent the first report of a defective human apolipoprotein, represents what will undoubtedly become the first of many reports in which a dyslipoproteinemia may be ascribed to a molecular defect in a plasma apolipoprotein.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02003-09 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Biochemistry and Metabolism of Plasma Lipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
	Richard E. Gregg, M.D.	Clinical Associate	MDB	NHLBI
	Leslie L. Jenkins, M.S.	Biologist	MDB	NHLBI
	Lila Taam	Chemist	MDB	NHLBI
	Elizabeth A. Rubalcaba	Chemist	MDB	NHLBI

COOPERATING UNITS (if any) Robert Scow, M.D., LNE, NIADD; Frank T. Lindgren, Ph.D., Donner Laboratory, University of CA, Berkeley, CA; Petar Alaupovic, Ph.D., Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.8	1.8	2.0

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Research in our laboratory has focused on the composition and metabolism of plasma lipoproteins and apolipoproteins (apo) in normal and dyslipoproteinemic man. Over the past 12 months we have completed analysis of radiolabeled apoA-I and apoA-II studies. These proteins are major constituents of human high density lipoproteins (HDL). A preliminary multicompartmental model for the metabolism of these proteins in man has been developed. Research on the interaction of radiolabeled apoA-I and apoA-II with native apolipoproteins and HDL has been completed. Radiolabeled apoC-II and apoC-III_2 studies have been completed in normal subjects. The metabolism of apoE, isolated from normal and dyslipoproteinemic patients (type III and type V hyperlipoproteinemia) has been studied in normal and type III patients. ApoC-II, apoC-III_2 and apoE are protein constituents of very low density lipoproteins (VLDL) and HDL. Analysis of human thoracic duct lymph lipoprotein and apolipoprotein 24 output data has been completed, as have incubation studies with lymph chylomicrons and lipoprotein lipase. In addition the effects of high cholesterol and high fat diets on plasma lipoproteins in normal subjects has been studied.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

1) To complete studies on apoA-I and apoA-II metabolism in normal and dyslipoproteinemic man.

2) To elucidate the interrelationship between lymph lipoproteins and plasma lipoproteins in man.

3) To define the plasma kinetics of apoC-III, apoC-III_2 and apoE in normal and dyslipoproteinemic subjects.

4) To determine the effects of various diets and drugs on plasma lipoprotein composition in man, and to study the clinical features of dyslipoproteinemic patients.

Methods Employed:

Methodology for the isolation, quantitation, and radioiodination of plasma lipoprotiens and apolipoproteins has been previously described. Methodology for plasma apolipoprotein determinations have been developed by other members of our branch. We are in the process of reimplementing a computerized record keeping system for purposes of storing and analyzing clinical and lipoprotein data on our patients.

Major Findings:

1) Apolipoproteins A-I and A-II are the major protein constituents of HDL. Previous studies from our laboratory have indicated that lymph chylomicron apoA-I and apoA-II can serve as precursors for these constituents within HDL. Immunologic studies on human jejunal mucosa performed by members of our branch are consistent with the concept that the human intestine can synthesize both apoA-I and apoA-II. Analysis of 24 hour output and lipoprotein distribution in human thoracic duct lymph indicates that approximately 20% of apoA-I and 15% of apoA-II in lymph is found associated with chylomicrons and VLDL, with the remainder being associated with lymph HDL. We estimate that the human intestine may contribute about 30-70% of total body apoA-I and apoA-II synthesis. Newly synthesized intestine apoA-I and apoA-II appears to enter the venous circulation from the lymph both on chylomicrons, VLDL, and HDL. In addition a significant amount of the HDL found in lymph appears to be dreived from the plasma. Therefore the human intestine appears to play a major role in human apoA-I and apoA-II synthesis.

2) When radiolabeled human lymph chylomicrons are incubated with lipoprotein lipase in vitro in the presence of albumin, apoA-I and apoA-II radioactivity is transferred to the HDL density region, apoB radioactivity is transferred to IDL (density 1.006-1.019 g/ml) and LDL (density 1.019-1.063 g/ml), and apoC-II and apoC-III radioactivity was transferred to IDL, LDL, HDL, and the 1.21 g/ml infranate. In these experiments over 90% of the

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chylomicron triglyceride was hydrolyzed to free fatty acid, and the bulk of lymph chylomicron cholesterol and phospholipid was transferred to LDL and HDL. It should be noted that lymph chylomicron apoB appears to consist in large measure in a lower molecular weight from that found in normal plasma LDL. The data are consistent with the concept that chylomicron degradation products can contribute to LDL and HDL protein and lipid mass.

3) In human plasma almost all apoA-I and apoA-II is found within the HDL density region (1.063-1.21 g/ml). This density region has been further subdivided into HDL_{2b} (1.063-1.10 g/ml), HDL_{2a} (1.10-1.125 g/ml) and HDL_3 (1.125-1.21 g/ml). HDL_{2b} and HDL_{2a} have higher apoA-I: apoA-II ratios then does HDL_3. Fluctuation in HDL levels are largely due to alterations in HDL_{2b} and HDL_{2a}. When HDL was infused into a patient with homozygous familial HDL deficiency (Tangier disease), the half lives for HDL_{2b}, HDL_{2a}, and HDL_3 were 0.1, 0.8, 2.7 days respectively, and there may have been some conversion of HDL_{2b} and HDL_{2a} to HDL_3. In this study apoA-I was catabolized at a significantly faster rate than apoA-II. Similarily in 14 normal subjects the plasma residence time of apoA-I was significantly lower than that of apoA-II. These data are consistent with the concept that HDL_{2b} and HDL_{2a} are catabolized at a more rapid rate than HDL_3, and that apoA-I is cleared from plasma more rapidly than apoA-II.

4) Previous data from our laboratory indicated that estrogen administration results in increased HDL protein synthesis in normal females. ApoA-I and apoA-II synthesis rates in normal females are higher than in their male counterparts, while fractional catabolic rates are similar. Therefore increased synthesis appears to account for the higher plasma apoA-I and apoA-II concentrations in females (as compared to males). However within each sex group, plasma apoA-I and apoA-II levels were not correlated with synthesis rate but residence time. These data suggest that estrogens may cause an increase in the synthesis of apoA-I and apoA-II, and that hormonal influences account for male-female differences with regard to apoA-I and apoA-II plasma levels and synthesis rate. The data also indicate that excluding the sex effect, alterations in the plasma level of apoA-I and apoA-II may relate to changes in the fractional catabolic rates of these two proteins rather than to their synthesis rates.

5) Previous data from this laboratory indicate that patients with familial HDL deficiency (homozygotes and heterozygotes), type I and V hyperlipoproteinemia, and abetalipoproteinemia all have decreased apoA-I and apoA-II, plasma levels, and enhanced fractional catabolism of these proteins. Based on normal and patient kinetic data, a multicompartmental model has been developed for both apoA-I and apoA-II metabolism (see annual report of Dr. Loren Zech).

6) Utilizing methodology and protocols previously developed in this laboratory, apoC-II and $apoC\text{-}III_2$ kinetics were studied in 7 normal subjects, and apoE kinetics were studied in 9 normals and 5 type III subjects. The mean residence time for $apoC\text{-}III_2$ (0.90 days) was slightly faster than that for apoC-II (1.13 days). ApoE was catabolized at a slower fractional rate

in type III subjects than in normals. (see annual report of Dr. Richard Gregg).

7) Ongoing diet studies over the past year in normal subjects indicate that use of high cholesterol (1000 mg/1000 calories/day) or high fat (64% fat) diets result in little change in plasma cholesterol, triglyceride, lipoprotein triglyceride, or apolipoprotein levels. The baseline diet in these studies was a 16% protein, 42% carbohydrate, 42% fat, 200 mg cholesterol/1000 calories/day, normal polyunsaturated: saturated fat ratio (0.1-0.3). However when the high cholesterol and high fat components were combined a significant increase in plasma apoB and cholesterol, and LDL cholesterol levels were noted, These data suggest that increased fat intake may augment cholesterol absorption resulting in alterations in LDL.

8) The clinical manifestations of familial HDL deficiency (Tangier disease) have been of increasing interest in view of HDL's putative role as a protective factor against atherosclerosis. The clinical features were reviewed and coronary heart disease (CHD) prevalence assessed in 27 Tangier homozygotes and 22 Tangier heterozygotes. No CHD was found in subjects under 35 years of age. In subjects between 35 and 65 years of age, 5 of 19 heterozygotes (26%) and 5 of 11 homozygotes (45%) had some evidence of CHD as compared to 4% or less in a normal population. The data are consistent with the concept that patients with Tangier disease may be at increased risk for CHD, but that this risk is not nearly as striking as that seen in homozygous familial hypercholesterolemia.

9) Patients with type V hyperlipoproteinemia frequently complain of dry eyes, dry mouth, and arthritis. The results of rheumatologic testing in such patients indicated that their clinical features were consistent with the sicca syndrome but that none had true Sjorgen's disease. As of July 1, 1980 clinical and laboratory data in normal and dyslipoproteinemia subjects will be placed on computer files, allowing for more facile analysis of the relationship between lipoprotein levels and various disease states.

Significance to Biomedical Research and the Program of the Institute:

HDL cholesterol has been shown to be inversely correlated with the incidence of CHD, while LDL cholesterol has been positively correlated with CHD. Factors regulating the concentration of LDL and HDL protein and lipid are therefore of great interest. The data discussed above provide insights into the regulation of LDL and HDL metabolism, and how these lipoproteins may relate to atherosclerosis.

Proposed Course:

Continuing objectives include study of apolipoprotein metabolism (apoA-I, apoA-II, apoB, apoC-I, apoC-II, apoC-III, apoE) in normal and dyslipoproteinemic subjects. These studies are of fundamental importance in our understanding of lipid transport and metabolism in normal individuals, and in patients with disorders of lipid metabolism and atherosclerosis. In addition we plan to study patterns of inheritance and clinical features in dyslipoproteinemic patients, and modalities (diet and drug) whereby such patients can best be treated.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02010-09 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure and Function of Plasma Lipoproteins and Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
Other:	Fairwell Thomas, Ph.D.	Research Chemist	MDB	NHLBI
	Rosemary Ronan, B.A.	Chemist	MDB	NHLBI
	Linda Kay, B.S.	Chemist	MDB	NHLBI
	Martha Meng, B.S.	Chemist	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Moleuclar Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.3	1.3	3.0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

ApoE have been isolated in electrophoretically homogeneous form from normal subjects and patients with Type III and V hyperlipidemia. ApoE from Type III patients was missing the E_3 isoform on isoelectric focusing, however on 2 dimensional electrophoresis the entire protein appeared shifted in pI. ApoE from all sources had identical amino acid compositions, however metabolic studies have shown that Type III E was functionally abnormal when compared to normal apoE.

ApoH has been isolated to electrophoretic homogeneity from lipoproteins of thoracic duct lymph and the chylo-VLDL fraction ($d<1.006$ g/ml) of normal and Type V subjects. ApoH was shown to be structural identical to β_2-glycoprotein-I. ApoH was shown to activated post heparin lipoprotein lipase, and is proposed to have an important role in triglyceride metabolism.

ApoB has been recognized to exist in man in two distinct eletrophoretic forms varying in apparent molecular weight on SDS gel electrophoresis. The larger and smaller apoB have been proposed to be of liver and intestinal origin respectively.

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PHS-6040
(Rev. 10-76)

Project Description

Objective:

1) Isolation and characterization of apoE.

The methods for the isolation of plasma apolipoproteins have been detailed in previous reports. The overall objective of these procedures has been the development of affinity chromatographic techniques for the isolation of apo-lipoproteins since these methods are rapid and have a relative high yield. ApoE has been purified to homogeneity from normal subjects and patients with phenotypes III and V. ApoE was isolated from plasma lipoproteins (d<1.006 g/ml) by heparin affinity chromatography followed by fractionation on Sephacryl S-200.

Major Findings:

ApoE obtained from the d<1.006 g/ml density fraction contained 3 major bands on isoelectric focusing. Patients with Type III hyperlipidemia were missing the $apoE_3$ band on one dimensional isoelectric focusing.

ApoE isolated by affinity and gel permeation chromatography was electrophoretic homogeneous on SDS gel electrophoresis. The apparent molecular weight by SDS gel electrophoresis was 32-34,000. ApoE dimers and apoE-A-II were also isolated. Isoelectric focusing of apoE from normal and patients with Type V hyperlipidemia were identical and revealed 3 major bands.

Of particular interest was the apoE isolated from patients with Type III hyperlipoproteinemia. ApoE isolated by these techniques was deficient in $apoE_3$ on isoelectric focusing. On 2 dimensional electrophoresis (isoelectric focusing x SDS gel electrophoresis) Type III apoE appeared shifted with all major polymorphic forms visualized. The amino acid analysis of normal, Type V and Type III apoE were v-rtually identical.

These combined results were interpreted to indicate that apoE isolated from patients with Type III was electrophoretically different from that of patients with Type V hyperlipidemia and normal subjects. The reason for the difference is as yet unknown and maybe either a difference in primary structure of the protein or a change in the post translational modification of the protein. Of particular interest were the metabolic studies carried out with normal, Type III, and Type V apoE. These studies established for the first time a functional defect in a plasma apolipoprotein (see annual report of Dr. Richard Gregg).

Objective:

2) Isolation and characterization of apoH.

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Methods Employed:

ApoH was isolated from human thoracic duct lymph and from lipoproteins of density < 1.006 g/ml of normal subjects and patients with Type V hyperlipidemia. ApoH was purified by heparin affinity chromatography followed by Sephacyl S-200 gel permeation chromatography.

Major Findings:

ApoH was isolated in electrophoretically homogeneous form from human thoracic duct lymph and chylo-VLDL lipoproteins of normal and patients with Type V hyperlipoproteinemia. The apolipoprotein was a single band on SDS gel electrophoresis with an apparent molecular weight of approximately 50,000. The amino acid analysis of apoH was similar from all sources. Detailed characterization revealed that the new apolipoprotein was different from any previously characterized apolipoprotein.

Antibodies prepared against apoH and the amino acid analysis of apoH revealed that the apolipoprotein was identical to β_2-glycoprotein-I. β_2-glycoprotein-I was one of several glycoproteins initially characterized in the 1950's as glycoprotein constituents of human plasma.

Of major importance was the finding that β_2-glycoprotein-I activated lipoprotein lipase. The activation of lipoprotein lipase was maximal in the presence of apoC-II (see annual report of Dr. Yoko Nakaya). Since β_2-glycoprotein-I had an affinity for lipid, was isolated on lymph and plasma, and activated lipoprotein lipase β_2-glycoprotein-I for convenience and in accord with the alphabetized nomenclature was designated apoH.

Objective:

3) Isolation and characterization of apolipoprotein B from plasma and thoracic duct lymph.

Methods Employed:

It has recently been recognized that two apoB proteins designated $apoB_L$ (large) and $apoB_S$ (small) can be visualized on SDS gels of plasma and lymph lipoproteins of density < 1.006 g/ml. These two apolipoproteins are presumed to be two forms of apoB since they have been reported in the rat to show immunocross reactivity and to have a similar amino acid composition.

Major Findings:

Antibodies to $apoB_L$ and $apoB_S$ have been prepared from apolipoproteins fractionated on SDS gel electrophoresis. Antibodies were prepared from a protein-gel admixture in both rabbits and goats. Monospecific antibodies to $apoB_L$ and $apoB_S$ have been prepared from the human apoB proteins. A detailed characterization of $apoB_L$ and $apoB_S$ including molecular weight, amino acid composition, and distribution within plasma and lymph lipoproteins is underway.

Objective:

4) Development of improved techniques for the automated sequence analysis of peptides and proteins.

Methods Employed:

Modifications of the Beckman 890D sequencer have been continued and included the use of a large reaction cup and cold trap. The details of the modifications and the preliminary results are included in last years report.

Major Findings:

The large reaction cup with a 75 percent increase in surface area and the addition of the cold trap have significantly improved the repetitive yield and decreased the nonspecific cleavage of protein and peptides degraded on the modified sequencer. Sequence analysis of several peptides and proteins including sperm whale myoglobin, insulin, and parathyroid hormone have definitively demonstrated improved yield as well as PTH derivatives with less ambiguity on analysis. These two modifications have become available as accessory units for the Sequencer by Beckman Instruments Company, Palo Alto, California.

Objective:

5) Identification of Phenylthiohydantoin (PTH) amino acids obtained during the automated degradation of peptides and proteins.

Methods:

Finnigan quadripole mass spectrometer equipped with PDP/8 computer.

Major Findings:

During the last several years we have been involved in the analysis of PTH amino acids by mass spectrometry. Extensive experience with this method has now permitted a detailed compilation of major ions as well as fragmentary ions which are associated with each PTH amino acid. This method has been uniquely suited to the identification of serine, threonine, glutamine, and asparagine. The mass spectrometric method is also the most sensitive as well as the fastest (< 2 minutes per PTH derivative) technique currently available for PTH analysis. The major ions used for this analysis have now been compiled for use by other investigators involved in protein structural analysis.

Significance to Biomedical Research and the Program of the Institute:

The isolation, characterization, and sequence analysis of the human plasma apolipoproteins is an ongoing program designed to ultimately understand the physiological role and molecular mechanisms involved in the synthesis, transport, and metabolism of plasma lipoproteins in normal individuals and patients with disorders of lipid transport and atherosclerosis.

Proposed Course:

The isolation, characterization, and structural analysis of plasma apolipoproteins within human thoracic duct lymph and plasma will be continued. Major emphasis will be continued on apolipoproteins C-II, D, H, and E. The ultimate understanding of the molecular defect in apoE in type III patients will be of major importance in our understanding of the role of apoE in lipid metabolism. The identification of apoH as a cofactor in the modulation of the enzymic activity of lipoprotein lipase will provide new insights into the regulation of triglyceride metabolism. The continued elucidation of the structure and function of plasma apolipoproteins will be a prerequisite to our ultimate understanding of the molecular mechanisms involved in lipid transport and metabolism.

Publications:

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2. Brewer, H. B., Jr., Schaefer, E. J., Zech, L. A., Osborne, J. C., Jr: Human plasma lipoproteins: Structure, function, and metabolism in lipoproteins and coronary heart disease, H. Greten, P. D. Lang and G. Schettler; editors, Gerhard Witzstrock Publishing House, New York, 1980, pp. 7-15..

3. Brewer, H. B., Jr., Schaefer, E. J., Osborne, J. C., Jr., Zech, L.A.: High density lipoproteins: An overview. NIH Publication No. 79-1661, pp. 29-41, 1979.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02011-05 MDB

PERIOD COVERED
October 1, 1979 through September 31, 1980

TITLE OF PROJECT (80 characters or less)

Molecular Properties of Lipoproteins and Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	James C. Osborne, Jr., Ph.D.	Senior Investigator	MDB	NHLBI
Other:	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI
	Thomas Bronzert, B.S.	Chemist	MDB	NHLBI
	Grace Huff, B.S.	Chemist	MDB	NHBLI
	Nancy Lee, M.S.	Chemist	MDB	NHBLI
	Alan Pocinki	Summer Student	MDB	NHBLI
	Rosemary Ronan, B.A.	Chemist	MDB	NHLBI
	Elizabeth Rubalcaba, B.A.	Chemist	MDB	NHLBI

COOPERATING UNITS (if any)

Luigi Servillo, University of Naples, Italy

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	2.0	4.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

These projects are directed towards a greater understanding of the quaternary organization of plasma lipoproteins and of the function of the oligomeric species involved in the transport and metabolism of lipids in plasma. The apolipoprotein composition of plasma lipoproteins is viewed as the governing factor in directing lipoprotein metabolism. Specificity is believed to be related directly to apolipoprotien secondary, tertiary and quaternary structure. Studies of the hydrodynamic properties of "lipid free" apolipoproteins, have been extended to include nitrated, sulfenylated and iodinated apolipoproteins in order to screen modification techniques commonly used for investigating lipoprotein metabolism. Analysis of the molecular properties of the specific self-associated and mixed-associated oligomeric apolipoprotein complexes (modified and native) are of major importance to our ultimate understanding of plasma lipoprotein metabolism.

Project Description

Objective:

1) Evaluation of the Mechanism of VLDL-HDL exchange of the C-apolipoproteins and the role of these apolipoproteins in the quaternary structure of VLDL and HDL.

In previous reports we have delineated the framework used in our laboratory to facilitate discussion of the role of apolipoproteins in the quaternary organization of plasma lipoproteins. Briefly, the apolipoprotein composition of plasma lipoproteins is governed through the laws of mass action by the affinity of specific apolipoprotein and/or lipoprotein species for different lipoprotein particles. With metabolism the lipoprotein particles are modified covalently, changing their affinity for specific apolipoprotein and/or lipoprotein complexes, which results in a redistribution of apolipoproteins. The role that lipid-free species play in these exchange/transfer processes is not known. In order to quantitate these interactions we have undertaken a systematic investigation of the solution properties of apolipoproteins. Previous reports have stressed the self-association of apolipoproteins in aqueous solution with particular emphasis on the major conformational changes that are concomitant with association. Apolipoproteins also undergo specific mixed-associations with other apolipoproteins. The analysis of mixed-associations between two self-associating proteins is simplified greatly if the spectral properties of the two apolipoproteins are different from one another. We have described previously our studies using modified apoA-II (the tyrosine residues were mono-nitrated with tetranitromethane). Studies with this probe were most consistent with the formation of the following mixed oligomers in aqueous solution:

apoA-II : apoA-I, 1:1 and 2:2
apoA-II : apoC-I, 2:4

Over the past year we have investigated the interaction between apoC-I and apoA-II using modified apoC-I.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

The mixed-association between apoA-II and apoC-I was evaluated using native apoA-II and labeled apoC-I. Since apoC-I contains no tyrosine and only one tryptophan residue we have modified this apolipoprotein by sulfenylation with sulfenyl chloride. As was found previously for nitrated apoA-II, the molecular properties of sulfenylated apoC-I (s-apoC-I) were quite similar to the native species except that the absorption spectrum was "red shifted" well above 300 nm with a maximum at 365 nm. Both native and sulfenylated apoC-I self-associate according to a monomer-dimer-tetramer scheme, both undergo indistinguishable major conformational changes that are concomitant with association and both cross react equally with antibodies prepared against unmodified apoC-I.

The distribution at equilibrium of s-apoC-I, in the model E ultracentrifuge, in the presence and absence of apoA-II was obtained by absorbancy measurements at 365 nm. Since only those species containing s-apoC-I contribute to the data at 365 nm the equation describing the equilibrium reduces to:

$$\begin{aligned}
\xi(r) &= C_{01}(m)\ \varepsilon^{A_{01}M_{01}(r^2-m^2)} \\
&+ K_{02}(m)\ C_{01}(m)^2\ \varepsilon_{01}\ e^{A_{02}M_{02}(r^2-m^2)} \\
&+ K_{04}(m)\ C_{01}(m)^4\ \varepsilon_{01}\ e\ A_{04}\ M_{02}^{\ (r^2-m^2)} \\
&+ \sum_{i=1}^{n}\sum_{j=1}^{m} K_{ij}(m)\ C_{10}(m)^i\ C_{01}(m)^i\ \frac{iM_{01}\ \varepsilon_{01}}{iM_{10}+jM_{01}} \\
&\mathrm{X}\ \ e^{A_{ij}M_{ij}(r^2-m^2)} \qquad (1)
\end{aligned}$$

As was found for nitrated apoA-II and native apoC-I, summarized above, a simultaneous analysis of data obtained at 280 nm at 365 nm was most consistent with a single mixed oligomer containing 2 molecules of apoA-II and 4 molecules of s-apoC-I with $K_{24} = 22{,}977 \pm 50\ (1/\mathrm{gm})^5$.

With a knowledge of the stoichiometry of the mixed interaction, we analyzed the profiles obtained with several different mixtures of native apoA-II and apoC-I according to basic equations. The resulting value of K_{24} was $31{,}248 \pm 890\ (1/\mathrm{gm})^5$. These combined data demonstrate experimentally that apoA-II and apoC-I apecifically interact in aqueous solution to form a mixed oligomer containing two molecules of apoA-II and four molecules of apoC-I.

Objective:

2) Evaluation of the effect of pressure on the molecular properties of

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apolipoproteins, lipoproteins and lipoprotein particles.

The effects of pressure on interacting systems depends upon the change in molar volume and is detected most easily in systems where there is a large change in molecular weight and/or the degree of exposure of nonpolar groups to solvent. In previous reports we have summarized the effects of pressure on apoA-I, apoA-II and apoC-I. Each of these apolipoproteins self-associates with major concomitant changes in secondary structure. ApoA-I self-associates according to a monomer-dimer-tetramer-octamer scheme. Therefore the molecular weight of the largest oligomer in solution is 224,128 and the effects of pressure on this system are demonstrated readily in the model E ultracentrifuge by monitoring the effects of rotor speed on the computed weight average molecular weight. The volume change for this system obtained from sedimentation equilibrium measurements is 12,327 ml/mole. The association scheme for apoA-II is monomer-dimer, and the molecular weight of the highest oligomer in solution is 34,760. Although the effects of rotor speed on the weight average molecular weight of apoA-II are not as dramatic as those found for apoA-I, we were able to calculate a molar volume change upon dimerization of 348 ml/mole from sedimentation equilibrium measurements. The effects of rotor speed on the weight average molecular weight of apoC-I were summarized in the last report. At that time we could not differentiate between monomer-dimer-trimer and monomer-dimer-tetramer association schemes for this system. The estimated volume change for this system was 0.032 ml/gm. Over the past year we have extended our investigations of this system in order to differentiate between monomer-dimer-trimer and monomer-dimer-tetramer association schemes for this system and to refine our estimates of the molar volume change upon oligomer formation.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

Major Findings:

In order to distinguish between monomer-dimer-trimer and monomer-dimer-tetramer association schemes for apoC-I, data must be obtained under conditions favoring the oligomer species. For ideal systems in which there is a positive volume change upon association these conditions are:

1) High total protein concentration which favors the oligomeric species through the laws of mass action.

2) Low rotor speed, in order to obtain data at low pressures which should favor the species with the larger partial specific volume.

Data were obtained at rotor speeds of 15,000, 20,000, 26,000 and 44,000 Rpm (15,000 is the lower limit of rotor speeds that can be employed reproducably for these low molecular weights) at initial concentrations varying from 0.2 to 3.15 mg/ml. The higher concentrations were analyzed by absorbancy measurements at 310 nm. Molecular weights were obtained for protein concentrations as high as 8 mg/ml using this technique. Two important features of the apoC-I systems emerged from an analysis of these data. First, the weight average molecular weight at the highest protein concentrations examined exceeded that of a trimer of apoC-I. This was especially evident if one takes into account that the weight average molecular weight obtained depends upon the value of the partial specific volumes used in the calculations. If the partial specific volume of the oligomer is taken to be 0.775 ml/gm, the value obtained from a statistical analysis of the effect of rotor speed on the weight average molecular weight of apoC-I, then the highest molecular weight obtained, 25,255 approaches that of tetrameric apoC-I, 26,520 and is much higher than trimeric apoC-I, 19,890. Thus over a larger concentration range, the monomer-dimer-tetramer association scheme is the only model tested that was consistent with the experimental data. The second feature of the data was that the weight average molecular weight obtained at a given concentration of protein *decreased* with *increasing* initial concentration of protein. This result was opposite to that expected for an ideal self-associating system where the partial specific volume of the oligomers is higher than that of the corresponding protomers.

Combining all data, the weight average molecular weight of apoC-I:

1) Decreases with increasing rotor speed.
2) Decreases with decreasing column height.
3) Decreases with increasing initial concentration of protein.

The first effect can be accounted for by postulating that the oligomers of apoC-I have a larger partial specific volume than the corresponding protomers. The latter two effects seem to be related to the concentration of protein at the meniscus of the cell. The data were analyzed therefore according to the following equation:

$$C = C_{1m}e^{A_1M_0}(r^2-m^2) + PC_{1m}$$

$$+ K_{12}(m)C_{1m}^{2}e^{2A_2M_0}(r^2-m^2)$$

$$+ K_{14}(m)C_{1m}^{4}e^{4A_2M_0}(r^2-m^2)$$

The value of P, which is related to the affinity of apoC-I for an air water interface was 19.4 ± 0.4 1/gm and the equilibrium constants at atmospheric pressure for dimer and tetramer formation were 17.3 ± 2.4 1/gm and 1386 ± 192 $(1/gm)^3$ respectively.

Objective:

3) Evaluation of the role of apolipoproteins in the quaternary organization of HDL.

The interaction between apolipoproteins and phospholipids in plasma lipoproteins has been the subject of numerous investigations over the past decade. Currently the most widely discussed and accepted model for this interaction involves the formation of amphipathic helical regions in the "lipid binding" portions of apolipoproteins. An amphipathic helix is defined most frequently as an α-helix in which there is a clear separation and partitioning of polar and non-polar residues such that the resulting polypeptide has a hydrophilic and hydrophobic surface. In this model the apolipoprotein in the lipid complex has very little, if any, tertiary and quaternary structure and the amphipathic helicies are oriented such that the nonpolar "face" interacts with non-polar side chains of the phospholipid micelles. The experimental evidence commonly used to stress the importance of amphipathic helicies in the interaction between apolipoproteins and lipids follows two lines: (1) the amino acid sequence of several apolipoproteins is such that amphipathic helical segments can be formed, using appropriate models, in portions of the polypeptide chains and (2) the secondary structure of apolipoprotiens increases, primarily in α-helical content, upon interaction with lipid. The fact that amphipathic helicies can be formed given the amino acid sequences of apolipoproteins is suggestive, but not unique to apolipoproteins; large portions of proteins such as myoglobin and glucagon, which are not integral components of plasma lipoproteins, can also be modeled theoretically into amphipathic helicies. Thus the amphipathic helical model of lipid-apolipoprotein interactions rests primarily on the increases in α-helical content in the presence of lipid. Unfortunately, these types of structural changes in apolipoproteins are not unique to lipids. We have summarized in previous reports our studies concerning the sensitivity of the secondary structure of apoA-I, apoA-II, and apoC-I to the presence of other ligands, such as inorganic phosphate. More importantly, the major structural changes that are concomitant with the self-association of these apolipoproteins makes it difficult to evaluate quantitatively the role of amphipathic helicies in lipid binding. In the past year we have extended our studies to include the effects of ligands on the secondary structure of apoC-III$_2$.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectrometer. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrometer and a Beckman Acta 3 spectrophotometer. Quaternary structure, i.e., intermolecular protein interaction, was studied by molecular weight measurements with a Spinco Model E ultracentrifuge and a Chromatrix Laser Light Scattering Photometer. Calculations and least square fits of the data to various models were performed with the MLAB program on the DEC system 10 computer at NIH.

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Major Findings:

ApoC-III_2 undergoes major increases in secondary structure in the presence of lipids. In contrast to apoA-I, apoA-II and apoC-I, there is little change in the secondary structure of apoC-III_2 with self-association. This observation simplifies the interpretation of lipid binding studies for this apolipoprotein. In order to address the uniqueness of lipid associated conformational changes we have extended our studies to other perturbants. The secondary structure of apoC-III_2 responds dramatically to inorganic phosphate. The α-helical content of apoC-III_2 increases from about 12% to over 36% in the presence of 1.5 M inorganic phosphate. Moreover, changes in secondary structure are observed at much lower concentrations, 0.01 molar; the effect of inorganic phosphate is a saturable phenomenon and corresponds to a langmuir isotherm. These findings, along with our previous studies, raise serious questions regarding the amphipathic helical theory of apolipoprotein-lipid interactions. The effects of inorganic phosphate on the secondary structure of apoC-III_2 are more dramatic than the corresponding effects of lipid. Moreover, changes in secondary structure are not unexpected when the environment of a protein is changed drastically by the addition of lipid; increases in secondary structure, primarily α-helical content, in non-polar environments are usually required in order to preserve hydrogen bonds. The effects of lipid on the secondary structure of apolipoproteins may be nonspecific and simply reflect the polarity of the new environment.

Objective:

4) Evaluation of the molecular properties of radiolabeled apolipoproteins.

Several laboratories, including the Molecular Disease Branch, have investigated the kinetics of the in vivo metabolism of radiolabeled apolipoproteins. The value of these data in terms of the metabolic fate of native apolipoproteins and lipoprotein particles depends directly upon any perturbations in the molecular properties of the resulting radiolabeled plasma lipoproteins caused by the labeling and/or incubation (recombination) procedure employed. The labeled apolipoprotein must mimic the distribution of the unlabeled species. We have begun an intensive comparison of the molecular properties of radiolabeled and unlabeled apolipoproteins A-I and A-II. The initial stages of this study involved a comparison of the elution profiles of labeled and unlabeled species from a 90 X 1.4 cm Sephadex column. Both apoA-I and A-II self-associate in aqueous solution and the elution profiles from columns are quite sensitive to the degree of association and thus initial (loading) concentration. The elution profiles of mixtures of radiolabeled (with ^{125}I; see last years annual report) and unlabeled apolipoproteins, labeled; unlabeled ~ 10^{-3}, were monitored by absorbancy and radioactivity measurements. Although the results were consistent with the participation of labeled apolipoprotein in the self-association of unlabeled species, the elution profile as measured by absorbance preceded that obtained by measurement of radioactivity for each apolipoprotein. This result was most probably due to a non-specific interaction between the column matrix and the radiolabeled apolipoprotein, however a more specific perturbation in the molecular properties of the radiolabeled apolipoprotein could not be ruled out at that time. Over the past year we have extended these studies to include the measurement of:

1) absorption spectra
2) circular dichroic spectra
3) Sephadex elution profiles at several different concentrations of protein.

Methods Employed:

Secondary structure, i.e., α-helical, β-pleated sheet, and random configurations, was investigated by using a Cary model 61 spectropolarimeter. Tertiary structure, i.e., the mode of intramolecular folding, was investigated by monitoring changes in the fluorescence and absorption properties on a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer and a Beckman Acta 3 spectrophotometer.

Major Findings:

The elution profile of apolipoprotein A-I from Sephadex G-100 columns (10 x 1.4 cm) depends critically upon the initial (loading) concentration of protein. At high concentrations the major peak corresponds to octameric apo A-I and with decreasing initial protein concentrations the elution profile shifts indicating slower migrating components and at low initial concentrations of protein the elution profile corresponds primarily to monomeric apoA-I. The elution profile of mixtures of native apoA-I and radiolabeled apoA-I (labeled: unlabeled ~ 6.5×10^{-5} to 2.2×10^{-3}) were obtained at high, > 1.7 mg/ml and low, < 0.05 mg/ml, initial concentrations of protein; previous studies were limited to intermediate, 0.4 mg/ml concentrations of protein where the elution profiles are quite complex. At low initial concentrations of protein, where the elution profile favors monomeric species, the peak of absorbancy preceded the corresponding peak of radioactivity. This result was consistent with that found at intermediate concentrations of protein and was attributed, in last years annual report, to possible nonspecific interactions between the labeled protein and the column matrix. In contrast, at high concentrations of protein, where the elution profile favors octameric apoA-I, the profiles of absorbancy and radioactivity were superimposable. This result is not consistent with a nonspecific interaction between apoA-I and the column matrix. Moreover, the elution volume obtained by radioactivity measurements did not exceed that expected for monomeric apoA-I even at the lowest concentrations of protein investigated. The circular dichroic and absorption spectra of the modified species at high concentrations of protein was indistinguishable from that of the native species. These combined results can be accounted for by two different mechanisms.

1) Iodination of apoA-I results in a decrease in the equilibrium constant for oligomer formation. In this case all labeled proteins participate in self-association with unlabeled proteins but the specific activity is higher in the monomeric species which results in a shift of the radioactivity

profile to favor slower migrating components.

2) Iodination of apoA-I results in the formation of an incompetent monomer, due to either double labeling of a single protein molecule or the labeling of a specific tyrosine residue. This incompetent monomer does not participate in self-association, which results in a higher specific activity of slower migrating components.

These two mechanisms will be evaluated in the coming year. It is important to stress that in either mechanism, the molecular properties of apo-A-I are modified by the labeling procedure. This may have major consequences in the use of radiolabeled apolipoprotein A-I to mimic the behavior of the corresponding unlabeled species.

Significance to Biomedical Research and Program of the Institute:

These investigations are directed toward a greater understanding of the quaternary organization and function of the plasma lipoproteins. The plasma lipoproteins are complex and their structure is sensitive to numerous perturbations including pressure, ionic strength and temperature. The apolipoprotein and lipid composition of plasma lipoproteins is related directly to the concentration and composition of other components of plasma, including other plasma lipoproteins. A quantitative knowledge of the organization and interactions of plasma lipoproteins is fundamental to our understanding of lipid transport and metabolism in normal individuals and in patients with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

Investigations concerning the molecular properties of apolipoproteins shall be expanded to include analysis of apolipoproteins E and H and quantitation of the mixed interaction between apoA-I and apoC-I. The studies of modified apolipoproteins shall be directed primarily to evaluating the perturbations produced by the iodination of apolipoprotein A-I. In addition, measurements of the surface activity of apolipoproteins shall be initiated. These studies continue to form a firm framework for our ultimate understanding of *in vivo* plasma lipoprotein interactions.

Publications:

1. Osborne, J. C., Jr., Brewer, H. B., Jr.: Solution properties of the plasma apolipoproteins. *Annals of The New York Academy of Science,* (In press).
2. Lloyd, M. A., Osborne, J. C., Jr., Safer, B., Huff, G., Merrick, W. C.: Characteristics of eukaryotic initiation factor 2 and its subunits, (1980), *J. Biol. Chem.,* 255: 1189-1193.
3. Osborne, J. C., Jr., Servillo, L: Equilibrium sedimentation of interacting systems: Mixed interaction between two self-associating

proteins. Biophysical Chemistry, (In press)

4. Brewer, H. B., Jr., Schaefer, E., Zech, L. and Osborne, J.C., Jr.: Human Plasma Lipoproteins: Structure, Function, and Metabolism, in Lipoproteins and Coronary Heart Disease, H. Greten, P. D. Lang and G. Schettler, editors, Gerhard Witzstrock Publishing House, New York, 1980 pg. 7-15.

5. Osborne, J. C., Jr.: Evaluation of the mixed association between two self-associating systems by sedimentation equilibrium, Biochim. Biophys. Acta, (In press).

6. Brewer, H. B., Jr., Schaefer, E. J., Osborne, J. C., Jr. and Zech, L. A.: High Density Lipoproteins: An Overview. NIH Publication No. 79-1661, August, 1979.

7. Servillo, L., Brewer, H. B., Jr. and Osborne, J. C., Jr.: Equilibrium Sedimentation of apolipoprotein C-I, Arch. Biochem. Biophys., (In press).

8. Servillo, L., Brewer, H. B., Jr. and Osborne, J. C., Jr.: Evaluation of the mixed interaction between apolipoproteins A-II and C-I by equilibrium sedimentation. Biophysical Chemistry, (In press).

9. Osborne, J. C., Jr., Powell, G. M. and Brewer, H. B., Jr.: Analysis of the mixed association between human apolipoprotein A-I and A-II in aqueous solution. Biochim. Biophys. Acta, (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02012-05 MDB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
In Vitro and In Vivo Regulation of 3-hydroxy-3-methylglutaryl Coenzyme A Reductase by Reversible Phosphorylation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Zafarul H. Beg, Ph.D.	Senior Investigator	MDB	NHLBI
Others:	John Stonik, B.S.	Chemist	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Rat liver 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) has been purified to homogeneity by a rapid procedure which employs HMG-CoA affinity chromatography. Antibodies prepared against purified HMG-CoA reductase gave a single immunoprecipitin line with HMG-CoA reductase. The in vitro phosphorylation of HMG-CoA reductase was studied utilizing purified enzyme and reductase kinase. With this system phosphorylated reductase contained approximately 4 mol phosphate per tetramer of 200,000. To demonstrate that HMG-CoA reductase undergoes phosphorylation in vivo, rats were injected with ^{32}P and hepatic reductase isolated by immunoprecipitation and by purification of the enzyme to homogeneity. Analysis of ^{32}P labeled immunoprecipitates and purified HMG-CoA reductase by SDS gel electrophoresis revealed a single peak of radioactivity comigrating with purified reductase establishing that reductase can undergo phosphorylation in vivo. Glucagon administration in vivo resulted in a 10 fold increase in hepatic cyclic AMP content, a two-fold increase in ^{32}P incorporation into HMG-CoA reductase, and a 40% decrease in HMG-CoA reductase enzymic activity.

PHS-6040
(Rev. 10-76)

Project Description

Objective:

1) In vitro phosphorylation of Homogeneous HMG-CoA reductase.

Methods Employed:

Rat liver HMG-CoA reductase was purified to homogeneity employing affinity chromatography on HMG-CoA. Reductase kinase was also purified to homogeneity. Purified HMG-CoA reductase was phosphorylated in the presence of radiolabeled ATP, $MgCl_2$ and purified reductase kinase.

Major Findings:

When purified HMG-CoA reductase was incubated with purified reductase kinase, ATP + $MgCl_2$, a time dependent inactivation of reductase activity was observed. Incubation of inactivated HMG-CoA reductase with phosphoprotein phosphatase was associated with a time-dependent increase in enzymic activity of reductase. These results are consistent with inactivation-reactivation of microsomal reductase reported earlier. Incubation of purified reductase with purified reductase kinase and γ-^{32}P-ATP + $MgCl_2$ was associated with a time dependent increase in protein-bound radioactivity and decrease in enzymic activity. The radiolabeled reductase was analyzed by SDS gel electrophoresis and radioactivity (>98%) was associated with the single electrophoretic band of purified reductase. Assuming that reductase is a tetrameter of 200,000 molecular weight, an average of 3.68 ± 0.56 mol of phosphate were incorporated per mol of tetramer. Treatment of [^{32}P]-labeled reductase with phosphatase resulted in a release of [^{32}P] radioactivity (>90%).

Objective:

2) In vivo phosphorylation of HMG-CoA redustase and its' modulation by glucagon.

Methods employed:

Rats were injected with [^{32}P], glucagon or saline under anaesthesia. Portions of livers were frozen in liquid nitrogen for the measurement of cyclic AMP and ATP content. Livers were used for the solubilization and purification of HMG-CoA reductase by affinity chromatography. Aliquots of partially purified enzyme were immunoprecipitated with a monospecific antibody to HMG-CoA reductase. [^{32}P]-labeled immunoprecipitates and purified HMG-CoA reductase were analyzed by SDS gel electrophoresis.

Major Findings:

Examination of [^{32}P]-labeled immunoprecipitates and purified enzyme by SDS gel electrophoresis revealed a single peak of radioactivity migrating with an apparent molecular weight of 51,000 (± 1800) coincident with the migration of purified HMG-CoA reductase. No immunoprecipitable radioactivity was detected on the gel when labeled HMG-CoA reductase was incubated with non-immunized goat serum. These results established that HMG-CoA reductase can undergo phosphorylation in vivo. The administration of glucagon was associated with a 40% decrease in the enzymic activity of HMG-CoA reductase and a 10 fold increase in hepatic cyclic AMP content. Dephosphorylation of HMG-CoA reductase from control and glucagon treated rats resulted in an increase in total enzymic activity to nearly identical levels. Analysis of [^{32}P]-labeled HMG-CoA reductase by SDS gel electrophoresis isolated by immunoprecipitation or by purification of enzyme to homogeneity revealed a two-fold increase in the [^{32}P] incorporation in glucagon treated over control rats. No change in ATP specific activity was observed following glucagon administration.

Objective:

3) Purification and characterization of cystolic cAMP-independent and microsomal cyclic AMP-dependent reductase kinase.

Methods Employed:

Cytosal obtained upon centrifugation of post-mitochondrial supernatant of rat liver was used for purification of the cAMP-independent reductase kinase. Ammonium sulfate fractionation (0-35%) followed by affinity chromatography on columns of affi-gel blue, Agarose-hexane-HMG-CoA and Agarose-hexane-ATP were employed. Microsomal cAMP-dependent reductase kinase was partially purified and required cAMP for it's activity.

Major Findings:

Purified cystolic cAMP-independent reductase kinase migrated as a single band on aqueous and SDS-gel electrophoresis, and had a monomeric and oligomeric molecular weight of 58,000 and 350,000 daltons respectively. Purified reductase kinase was able to catalyze the phosphorylation of purified HMG-CoA reductase. Partially purified cAMP-dependent microsomal reductase kinase also inactivated HMG-CoA reductase in the presence of ATP + $MgCl_2$ and cAMP. The inactivation of HMG-CoA reductase in the presence of cAMP was completely blocked by a muscle protein kinase inhibitor. Cytosolic reductase kinase appears to be similar to microsomal reductase kinase purified and characterized previously.

Significance to Biomedical Research and the Program of the Institute:

These studies are directed toward an understanding of the short-term regulation of the rate limiting enzyme of cholesterol biosynthesis HMG-CoA reductase. An elucidation of the mode of control of this enzyme will allow a detailed analysis of the parameters involved in the cellular regulation of cholesterol metabolism in normal subjects, and patients with atherosclerosis.

Proposed Course:

A systematic investigation of the modulation of HMG-CoA reductase and reductase kinase will be continued. The cAMP-dependent HMG-CoA reductase kinase will be further characterized. In vivo regulation of HMG-CoA reductase and reductase kinase under different physiological conditions (e.g. cholestyramine and cholesterol feeding, administration of other hormones, etc.) will be investigated during the next year. These studies will enhance our understanding of the role of this important enzyme in the cellular regulation of cholesterol synthesis.

Publications:

1. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: 3-hydroxy-3-methylglutaryl coenzyme A reductase from avian liver: catalytic properties. Biochim. Biophys. Acta. 572: 83-94, 1979.

2. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: Characterization and regulation of reductase kinase, a protein kinase which modulates the activity of HMG-CoA reductase. Proc. Natl. Acad. Sci. USA: 76, 4375-4379, 1979.

3. Beg, Z. H., Stonik, J. A., and Brewer, H. B., Jr.: In vitro and in vivo phosphorylation of rat liver 3-hydroxy-3-methylglutanyl coenzyme A reductase and its modulation by glucagon. J. Biol. Chem. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02015-03 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Isolation, Characterization, and Quantitation of Lipoproteins and Apolipoproteins from the Plasma and Lymph of Normal Individuals and Patients with Dyslipoproteinemia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David Anderson, Ph.D.	Staff Associate	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any) Dr. F. T. Lindgren, Donner Laboratory, University of CA, Berkeley, CA; Dr. Trudy Forte, Donner Laboratory, University of CA, Berkeley, CA; Dr. Thomas E. Starzl, Department of Surgery, University of CO Medical Center, Denver, CO; Dr. Gary D. Niblack, Renal Transplant Unit, VA Hospital, Nashville, TN

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.3	PROFESSIONAL: 1.3	OTHER: 0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The daily transport of human plasma apoA-I and apoA-II, triglyceride and total cholesterol from the thoracic duct lymph into plasma was measured in two subjects prior to and three subjects subsequent to renal transplantation. Lymph triglyceride transport was approximately 83% of the daily ingested fat loads while lymph cholesterol transport was consistently greater than the amount of daily ingested cholesterol. Lymph apolipoprotein transport exceeded the predicted apolipoprotein synthesis rate by an average of 659 ± 578 mgs/day for apoA-I and 109 ± 59 mg/day for apoA-II among the five subjects. Lymph HDL particles are mostly HDL$_{2b}$ and HDL$_{2a}$ and have a greater triglyceride and smaller cholesterol ester content when compared to homologous plasma HDL. The major quantity of both lymph apoA-I (81 ± 8%) and apoA-II (90 + 11%) was found within HDL with almost all of the remainder found in chylomicrons and VLDL. The combined results are consistent with a major contribution of the intestine to total body synthesis of apoA-I and apo-A-II. An important role of lymph in returning filtered apolipoprotein to plasma in association with HDL is proposed.

Project Description

Objective:

In collaboration with the scientists listed above, we undertook to quantitate the daily transfer of human lymph apolipoproteins (apo) A-I, A-II, triglyceride and total cholesterol from thoracic duct lymph into plasma, and to determine the distribution of these components among lymph lipoprotein classes. In addition, the composition and physical properties of lymph high density lipoprotein (HDL) are compared with those of plasma HDL.

Methods Employed:

Human thoracic duct lymph was obtained by cannulation of the thoracic duct in five subjects undergoing lymph drainage for purposes of immunosuppression either prior to kidney transplantation (2 subjects) or following transplantation (3 subjects). The latter 3 subjects were non-uremic. The concentrations of apoA-I and A-II were determined in whole lymph and lymph lipoprotein fractions by radial immunoassay and electro-immunoassay. The cholesteryl ester and total cholesterol content of all fractions was determined with the Beckman oxygen electrode-enzymic analyzer (T. J. Bronzert and H. B. Brewer, Jr., Clin. Chem. 23: 2089-2098). Phospholipid concentrations in lymph and plasma samples were determined as lipid phosphorus by the method of Chalverdjian. Total cholesterol and triglyceride concentrations were measured using the Autoanalyzer II. Total protein was determined by the method of Lowry. Electron microscopy of both whole lymph and lymph lipoprotein fractions was carried out utilizing the negative stain method.

Analytic ultracentrifugation of both lymph and plasma samples was performed and HDL_{2b}, HDL_{2a} and HDL_3 concentrations were determined (D. Anderson et al, Atherosclerosis 29: 161-179, 1978).

Major Findings:

Results obtained for uremic and nonuremic subjects were generally the same. In specific it was found that:

1) The daily transport of triglyceride represented approximately 83% of ingested fat, independent of fat load.

2) Lymph cholesterol transport varied between 0.75 and 3.10 gms/day and was noticeably greater than the estimated daily ingested load.

3) Lymph apoA-I and apoA-II transport was equal to and often significantly greater than the predicted apolipoprotein synthesis rate for all subjects. This finding is noteworthy in view of the large variation in volumes of lymph flow (25.1-112.2 dl/day).

4) The major quantity of both lymph apoA-I (73-95%) and apoA-II (73-100%) was found within HDL with the remainder in chylomicrons and VLDL.

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5) Lymph HDL concentrations were only a fraction of plasma HDL concentrations (8-20%). The proportion of lymph HDL which exhibits the faster flotation rates of HDL_{2b} and HDL_{2a} (79-100%) is greater than for plasma HDL (48-67%).

6) The weight percent triglyceride is higher and the weight percent cholesteryl ester lower in lymph HDL as compared with plasma HDL.

Significance to Biomedical Research and the Program of the Institute:

Human thoracic duct lymph contributes a major amount of apolipoproteins A-I and A-II to the plasma compartment. It thus may play a significant role in the homeostasis of normal lipid and lipoprotein metabolic pathways. The elucidation of these pathways in plasma and extraplasma fluids will facilitate our knowledge of lipid transport and catabolism in individuals with disorders of lipid metabolism and atherosclerosis.

Proposed Course:

The time course of lipid and apoprotein transport into the plasma via the thoracic duct lymph will be investigated on an hourly basis. In addition to apoA-I and apoA-II, the transport of apoC-II and apoB will be determined. The effect of meals and time of day on lipid and apolipoprotein transport will also be investigated.

Publications:

1. Anderson, D. W., Schaefer, E. J. Bronzert, T. J., Lindgren, F. T., Forte, T., Starzl, T. E., Niblack, G, D., Zech, L. A., and Brewer, H. B., Jr.: Transport of apolipoproteins A-I and A-II by human thoracic duct lymph. J. Clin. Invest. (In press).

2. Schaefer, E. J., Anderson, D. W., Zech, L. A., Lindgren, F. T., Bronzert, T. A., Rubalcaba, E. A., and Brewer, H. B., Jr.: The metabolism of high density lipoprotein subfractions and constituents in tangier disease following the infusion of high density lipoproteins. J. Lipid Res. (In press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02017-03 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Theoretical Analysis of the Metabolism of Lipoproteins and Their Apolipoprotein and Triglyceride Moieties

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any) Mones Berman, Ph.D., Laboratory of Theoretical Biology, NCI; Scott M. Grundy, M.D., Ph.D., Dan Steinberg, M.D., Ph.D., University of Calif., La Jolla, CA; Barbara Howard, Ph.D., Peter H. Bennett, M.D., Phoenix Clinical Research, Center Section, NIAMDI

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The plasma mass of apolipoproteins A-I and apolipoproteins A-II was examined in 14 normal individuals selected at random following 10 days of stabilization on a metabolic diet. ApoA-II had become stable in more than 80% of the subjects while A-I was stable in 60%. Using statistical models fasting and non-fasting determinations of apoA-II were found to be equal while a parallel examination of apoA-I mass found the non-fasting values to be elevated by one-half standard deviation. Triglyceride kinetics were studied in hyperlipidemic subjects utilizing radiolabeled glycerol before and following therapy with nicotinic acid. Analysis using a compartmental model revealed that nicotinic acid decreases the residence time of VLDL-triglyceride without a significant change in the absolute synthesis rate. Changes in triglyceride kinetics following drug treatment resulted in secretion of more VLDL or an unchanged number of VLDL containing more triglyceride but usually not both. Triglyceride kinetics examined in Pima Indians using a compartmental model were found to correlate with post-prandial plasma free fatty acid levels; synthesis rate $r=.76$ $p<.005$ a strict precursor product relationship between VLDL and LDL did not exist.

Project Description

1) Objective:

Development of both compartmental and statistical models for analysis of apoA-I and apoA-II metabolism in normal subjects and patients with disorders or lipoprotein metabolism, atherosclerosis, and other lipid abnormalities.

Methods Employed:

The methods used for the development of multicompartmental models using turnover data from radiolabeled apolipoproteins and plasma lipoprotein studies have been detailed in previous reports. These models are stimulated using the SAAM simulator (a large collection of digital computer programs on the Univac 1108 computer at the National Bureau of Standards, the Peripheral Data Processor 10 at the National Institutes of Health, and the VAX/780 digital computer in the Laboratory of Theoretical Biology, National Cancer Institute. These simulated results are compared to the experimental results and the connectivity (number and topology of compartments) as well as flow of the model changed until a working model is developed. Using this model the volume of distribution of the apolipoproteins is estimated and compared to independent estimes of these volumes. After development of the compartmental model, the parameters of the model are adjusted using nonlinear least square techniques resulting in a minimal least square error. These have now been extended to studies in which differences in multicompartmental models, developed using turnover data from two radiolabels on two different apolipoproteins in normal individuals, have been compared.

In addition, standard statistical methods have been used to develop and test statistical models developed to simulate changes in plasma apolipoprotein values following both short-term (hour to hour) and long-term (day to day) perturbations.

Major Findings:

1) Using plasma decay curves obtained from 21 day experiments residence times were determined for apoA-I and apoA-II. There was no change in the results from 21 day studies when compared to previous 14 day studies. No paired studies were examined.

2) Using both plasma, urine, and whole body data, the model developed to describe results from 14 day experiments was tested against results of long duration (21 day) kinetic experiments. Models of apoA-I in 4 subjects and apoA-II in 2 different subjects were adequate to explain 21 day kinetic data without change.

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3) Mean fasting apoA-I values were found to be one-half standard deviation decreased when compared to non-fasting samples. The variance in the fasting and non-fasting values was equal.

4) Mean fasting apoA-II values were found to be unchanged when compared to non-fasting values. The variance in the fasting and non-fasting distributions was also equal.

5) Plasma apoA-I levels were determined in randomly selected normal volunteers following stabilization for more than 14 days on a hospital, metabolic diet. More than 60% of the determinations had reached constant apoA-I mass values ($p<.05$). Plasma apoA-II mass values ($p<.05$) had become constant in the same 14 subjects.

2) Objective:

1. Continue development of a multicompartmental model for triglyceride metabolism and the precursors of triglyceride metabolism.

2. Estimation of the parameters describing triglyceride metabolism including synthesis rate, residence time, and fractional catabolic rate in a group of subjects with hyperlipidemia before and after treatment with lipid lowering drugs.

Methods Employed:

Radiolabeled glycerol was administered to patients with varying degrees of hyperlipidemia before and after treatment with the lipid lowering drug, nicotinic acid. The topological structure (connectivity and number of compartments) of multicompartmental models previously developed on this project was examined for their ability to explain the experimental results before and after treatment with nicotinic acid. After it was established that this model could explain the observed data it was used to calculate the kinetic parameters of VLDL-triglyceride metabolism of all subjects. In addition, differences in metabolism associated with administrations of nicotinic acid therapy for reduction in lipids was examined. Each subject acted as its own control.

Using changes in kinetic parameters it was possible for the first time to establish relative differences in lipoprotein size and number following drug treatment.

Major Findings:

1) Analysis of kinetics of very low density lipoprotein triglyceride kinetics was performed in collaboration with Dr. Mones Berman (Laboratory of Theoretical Biology, NCI), and Dr. Scott Grundy (Veterans Administration Hospital, La Jolla CA). Comparison of subjects with hyperlipidemia on and off drugs established that an increase in VLDL-TG fractional catabolic rate or conversely a decrease in residence time for VLDL-TG was a result of nicotinic acid therapy.

2) There was no consistent change in the synthesis rate of VLDL triglyceride following nicotinic acid therapy. Both increase, decrease, and no change was observed in this kinetic measurement.

3) Relative changes in very low density lipoprotein particle size and number were determined following nicotinic acid therapy. Subjects which made large VLDL particles decreased the size of nascent VLDL following therapy while subjects which made small VLDL particles decreased the relative number.

3) Objective:

Analysis of the very low density lipoprotein triglyceride kinetics in Pima Indians.

Methods Employed:

The Pima Indian nation of Native Americans are an interesting, well-defined group of subjects. Studies of this group were performed in collaboration with Dr. Barbara Howard and Dr. Peter Bennett (Phoenix Experimental Station, NIAMDD). These individuals are of interest because they have an increased incidence of diabetes, decreased incidence of lithogenic bile and cholesterol gallstones, and increased incidence of obesity greater than 150% of ideal body weight. In addition, they also represent a well-defined group in which insulin, glucose, cholesterol, and low density lipoprotein triglyceride kinetics have been previously studied.

Very low density lipoprotein triglyceride was endogenously labeled following the injection of 300 Ci of radiolabeled precursor, ^{3}H-glycerol. This method is considered to induce the least amount of artifact. In addition, plasma levels of insulin, glucose and free fatty acids were determined by standard techniques.

The kinetics of very low density lipoprotein triglyceride kinetics in normal triglyceridemic, non-diabetic, male, Pima Indians was examined using a compartmental model previously developed for triglyceride synthesis and metabolism. Using standard statistical techniques the relationships among several kinetic parameters (fractional catabolic rate, synthesis rate, delipidation rate, etc.) and plasma values of glucose, insulin and free fatty acids were examined in detail.

Major Findings:

1) Studies completed in 15 Pima Indians were of sufficient sample size to make statistical significant comparisons with previously examined groups.

2) The connectivity (number of compartments and the topology of tracer flow) of the model developed for the Pima Indians was equivalent to the model developed previously for caucasian males.

3) The kinetic parameters were compared with two groups composed of normal weight (125% ideal body weight) and obese (160% ideal body weight) normal triglyceridemic caucasians. The fractional catabolic rate for very low density lipoprotein triglyceride for Pima Indians was increased (.42 h^{-1}) compared to normal (.21 h^{-1}) and obese (.32 h^{-1}) caucasians. The synthesis rate in Pima Indians (803 mg/hr) was equal to normal (727 mg/hr) but less than obese caucasians (1414 mg/hr).

4) No kinetic parameter describing triglyceride kinetics was correlated with fasting, one hour, two hour or three hour post prandial glucose or insulin determinations.

5) The peak plasma free fatty acid level correlated well with synthesis rate $r=.76$ $p<.001$.

6) The very low density lipoprotein particle absolute catabolic rate exceeded previously reported low density lipoprotein synthesis rates. Therefore there is not a strict precursor product relationship between VLDL and LDL in these subjects.

7) Normal triglyceridemic, non-diabetic, male Pima Indians secrete very low density lipoproteins which are triglyceride rich when compared to normal caucasians.

Significance to Biomedical Research and the Program of the Institute:

Understanding the metabolism of lipoproteins and the moieties which make up these lipoproteins (cholesterol, cholesterol ester, triglycerides, and apolipoproteins) are significant because of their relationship to atherosclerosis and cholelithiasis. The development of a concept of plasma lipoprotein metabolism is approached by the theoretical analysis of data from metabolic studies using the techniques of compartmental and statistical model building. This type of theoretical analysis provides a framework for discussion between investigators. This project consists of the testing and further development of compartmental models for lipoprotein metabolism as well as the proposal of new models where they do not exist.

Understanding lipoprotein metabolism is of major importance due to the central role of lipoproteins in the transport and catabolism of cholesterol and triglycerides in normal and patients with disorders of lipid metabolism and/or atherosclerosis. Because of the recent elucidation of the negative correlations between HDL-cholesterol levels and the incidence of coronary heart disease the understanding of the two major HDL apolipoproteins (apoA-I and apoA-II) is particularly relevant to the understanding of atherosclerosis.

Understanding the modulation of lipoprotein, cholesterol, cholesterol ester, triglycerides and apolipoprotein by drugs, diet, and genetic disease is also of significance since changes in these modulators may have major effects on atherosclerosis and cholelithasis. This theoretical analysis also provides a framework for comparison between groups as dissimilar as caucasians and American Indians.

Proposed Course:

Detailed studies will be continued on the analysis of the differences in apoA-I and apoA-II metabolism by further specifying and defining the current compartmental models, with particular emphasis on analysis of paired studies of longer duration, and studies in which alternative methods of labeling are employed. Preliminary studies will be initiated on the analysis of apoE and apoC metabolism. The overall objective will be the development of a comprehensive model of human lipoprotein metabolism by the incorporation of this information into the previously proposed LpB model. The formulation of an overall conceptualization of lipoprotein metabolism in normal individuals and in subjects with disorders of lipoprotein metabolism will be continued by qualitative and quantitative testing of these conceptions using compartmental modeling and other theoretical methods. Of particular interest will be the determination of which parameters remain invariant and variant when the systems are modulated by diet, drug, and transformed by genetic disease.

Publications:

1. Howard, B. V., Zech, L. A., Davis, M., Brennion, L. J., Savage, P. J., Nagulesparan, M., Bilheimer, D., Bennett, P. H., Grudy, S. M.: Studies of very low density lipoprotein triglyceride metabolism in an obese population with low plasma lipids. J. Lipid Res., (In press).

2. Grundy, S. M., Mok, H. Y. I., Zech, L. A., Berman, M.: Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. J. Lipid Res., (In press).

3. Davies, P. J. A., Chabay, R., Zech, L. A., Berman, M., Pastan, I.: Prostagladin E_1 binding and adenylate cyclase activation in normal and transformed fibroblasts. Biochim. Biophys. Acta, 629: 282-291, 1980

4. Fisher, W. R., Zech, L. A., Bardalaye, P., Warmke, G., Berman, M.: The metabolism of apolipoprotein B in subjects with hypertriglyceredemia and poly disperse LDL. J. Lipid Res., (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02018-02 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Immunolocalization of Apolipoproteins in Human Tissue

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	David E. Schwartz, M.D.	Clinical Associate	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)
Renee Virmani, M.D., Armed Force Institute of Pathology, Robert Varnham, M.D., Pathology Department, Duke University, Durham, NC.

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.2	1.2	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The peroxidase immunocytochemical technique has been adopted for the localization of apolipoproteins in human tissue. Antibodies to apolipoproteins C-I, C-II, C-III, B, D, A-I, H, and E have been developed in the Branch. Several tissues have been obtained for analysis of apolipoprotein content. Human jejunem from Tangier patients was shown to contain apoA-I, apoA-II, and apoB. These results were consistent with data from metabolic studies which indicated that patients with Tangier disease were able to synthesize apoA-I, apoA-II, and apoB. The defect in this disease is therefore not due to defective synthesis of the apolipoproteins.

The coronary atherosclerotic lesions of patients with Type III hyperlipidemia was investigated and examined by the peroxidase technique. These lesions were similar to those observed in "typical" atherosclerotic lesions. Peroxidase staining revealed that the foam cells were positive for apolipoproteins A-I, A-II, B, C-I, and C-II. These studies have indicated that apolipoproteins associated with both LDL and HDL are present within the foam cells.

Project Description

Objective:

To determine the presence or absence of apolipoproteins and their cellular distribution in tissue from the intestine, liver, and coronary arteries in normal subjects and patients with dyslipoproteinemias.

Methods Employed:

The immunohistochemical technique reported by Sternberger (J. Histochem. Cytochem. 18, 315, 1970) utilizing peroxidase was modified and adapted for immunolocalization studies. With this technique unlabeled monospecific antibodies to specific apolipoproteins were employed to link horseradish peroxidase to a specific tissue apolipoprotein. A chromagen is then precipitated at this site via a redox reaction involving hydrogen peroxidase as the oxidizing agent.

Tissue samples were obtained in paraffin blocks from fresh tissue or previous surgical or autopsy specimens. These tissue samples are sliced on a cytrostat or microsome and transferred to gelatinized slides for peroxidase staining as well as staining with red O, movat, and hexatocylin/cosin.

Major Findings:

1) The intestine from normal subjects contained apoA-I, apoA-II, and apoB. The intensity of staining for all apolipoproteins increased at the villus tip, and was localized intracellularly particularly in the supranuclear Golgi region.

2) Intestinal biopsy samples from patients with Tangier disease showed a positive peroxidase staining for apolipoproteins apoA-I, apoA-II, and apoB. The pattern of staining for control subjects and Tangier patients was identical. These data are consistent with the view that Tangier patients are able to synthesize apoA-I, apoA-II and apoB in their intestinal mucosa despite strikingly low levels of these proteins in their plasma.

3) Foam cells in atherosclerotic plaques were shown to contain apolipoproteins A-I, A-II, C-I, C-III, and apoB. These foam cells were localized to the intimal and medial of the artery. These studies were interpreted to indicate that lipoproteins containing apoB, as well as HDL have been localized in foam cells. HDL are of recent interest since HDL-cholesterol has been recognized to be a negative risk factor for the development of premature cardiovascular disease.

4) The coronary atherosclerotic lesions in patients with Type III hyperlipidemia were similar to the lesions seen in patients with Type II hyperlipidemia. There was no evidence of a unique foam cell type of atherosclerosis in Type III patients.

Significance to Biomedical Research and the Program of the Institute:

The major sites of synthesis of plasma apolipoproteins are of major importance in our understanding of the biosynthesis and metabolism of plasma lipoproteins. The intestine has recently been recognized as a major source of plasma apolipoproteins. The application of the peroxidase technique will facilitate our understanding of the role of specific tissue in apolipoprotein biosynthesis.

The use of this procedure in the analysis of the apolipoprotein content of atherosclerotic lesions in normal lipidemia and dyslipidemic subjects will be of major importance in our detailed analysis of the molecular mechanisms involved in the atherosclerotic process.

Proposed Course:

Future research will be directed to a further analysis of the sites of synthesis of the human plasma apolipoproteins. In addition, studies of the origin and apolipoprotein content of foam cells in the atherosclerotic lesions will be continued.

Publications:

1. Schwartz, D. E., Farnham, R., Schaefer, E. J., and Brewer, H. B., Jr.: Immunolocalization of Apolipoproteins A-I, A-II, and B in Normal and Tangier Intestinal Mucosa. Cell. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02019-02 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism of Human Plasma Apolipoproteins

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Richard E. Gregg, M.D.	Clinical Associate	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	Loren A. Zech, M.D.	Senior Investigator	MDB	NHLBI
	Leslie L. Jenkins, M.S.	Biologist	MDB	NHLBI
	Lila Taam, B.S.	Biologist	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3.5	PROFESSIONAL: 2.0	OTHER: 1.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The metabolism of apolipoprotein E (apoE) has received major emphasis in the past year. ApoE is elevated in animals with experimental hypercholesterolemia and atherosclerosis. In addition, patients with Type III hyperlipoproteinemia (HLP) have an abnormal apoE, elevated levels of apoE, and accelerated atherosclerosis.

ApoE from normal subjects, Type V HLP patients (indistinguishable from normal apoE) and Type III HLP patients was purified, radioiodinated and injected into normal and Type III HLP patients. Type III HLP patients had 4 times the normal apoE level and the residence time for normal and Type V apoE was 2 times normal. Type III apoE had a prolonged residence time in both normal individuals and Type III HLP patients. These results inidcate that Type III HLP patients have a catabolic defect for Type V apoE. In addition they indicate that Type III apoE is abnormal and is metabolized at a slower catabolic rate in both normal individuals and patients with Type III HLP. These studies represent the first demonstrated molecular defect in an apolipoprotein which results in hyperlipidemia.

PHS-6040
(Rev. 10-76)

Project Description

Objectives:

These studies were performed to determine the metabolic parameters of normal apoE in normal individuals and Type III HLP patients. In addition, the metabolic parameters of abnormal Type III apoE were also determined. ApoE has been proposed to regulate the metabolism of the metabolic remnants of chylomicrons and these particles may be atherogenic. Animals with experimentally induced hypercholesterolemia have elevated levels of apoE and accelerated atherosclerosis. Patients with Type III HLP have elevated cholesterol and triglyceride levels, elevated apoE levels, an abnormal type of apoE, and accelerated atherosclerosis. This suggests that apoE is important in regulating lipoprotein metabolism and may also be important in the development of atherosclerosis. By studying the metabolic parameters of apoE metabolism, it is hoped that insights will be gained on how apoE exerts its influence on lipoprotein metabolism and atherosclerosis.

Methods Employed:

Normal subjects (N=11) and Type III HLP patients (N=5) were placed on an isocaloric balanced diet with a normal cholesterol level and normal P/S ratio. Normal, Type V or Type III apoE was radioiodinated by the iodine monochloride method and injected into the study subjects. Timed blood samples were obtained and the lipoprotien subclasses were separated by ultracentrifugation. ApoE levels were determined by electroimmunoassay and the radioactivity was determined in a gamma counter. The total amount of radioactivity remaining in the body was measured daily in a whole body counter. Plasma residence times were determined using a multiexponential computer curve fitting technique. Plasma volumes, apoE pool sizes, fractional catabolic rates, and production rates were calculated from the above data.

Major Findings:

1) Normal or Type V apoE is catabolized very rapidly in normal individuals with a residence time of about 0.34 days.

2) Type V apoE is catabolized much slower in Type III HLP patients with a residence time of about 0.62 days.

3) Compared to Type V apoE, Type III apoE is catabolized more slowly in both normal individuals and Type III HLP patients.

4) Type V apoE and Type III apoE have a significant portion of their total body distribution in nonplasma spaces in both normal individuals and Type III HLP patients.

5) The elevated apoE level in Type III HLP is due to a defect in catabolism of the abnormal Type III apoE. The role of increased synthesis has not been determined at this time.

Significance to Biomedical Research and the Program of the Institute:

Elevated levels of apoE are associated with experimental hypercholesterolemia with atherosclerosis, and Type III HLP with premature atherosclerosis. These studies have shown that the electrophoretically abnormal Type III apoE is of metabolic significance and may be the primary defect in Type III HLP. In addition, the methods have been developed and the control studies performed so that apoE metabolism in various other HLP syndromes can be investigated. These studies will give further insight into the mechanisms by which apoE may regulate lipoprotein metabolism and the development of atherosclerosis.

Proposed Course:

It is planned to continue the study of apoE metabolism in normal subjects and HLP patients. In addition, an apoE radioimmunoassay will be developed in the near future so apoE levels can be accurately quantitated in plasma and various lipoprotein subfractions.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02020-01 MDB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Apolipoprotein Modulation of the Enzymic Activity of Lipoprotein Lipase

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoko Nakaya, M.D., Ph.D.	Visiting Scientist	MDB	NHLBI
Other:	Ernst J. Schaefer, M.D.	Senior Investigator	MDB	NHLBI
	H. Bryan Brewer, Jr., M.D.	Chief	MDB	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Molecular Disease Branch

SECTION
Peptide Chemistry

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Lipoprotein lipase (LPL) is the major enzyme involved in the hydrolysis of triglycerides of VLDL and chylomicrons. LPL can be isolated by heparin affinity chromatography from human post heparin plasma. ApoC-II is a well known activator of the enzymic activity of LPL. ApoH is a recently described protein (β_2-glycoprotein-I) constituent of triglyceride rich lipoproteins in human lymph and plasma. ApoH has been shown to increase the enzymic activity of LPL in the presence of apoC-II by 45+/-17 percent. ApoC-III decreased the apoH and apoC-III enhanced activity by LPL by 77 percent. These results provide evidence that this new apolipoprotein, apoH, modulates the enzymic activity of LPL in triglyceride metabolism. The ultimate proportions of apoH, apoC-II, and apoC-III in triglyceride rich lipoprotein particles may determine the ultimate rate of LPL catalyzed triglyceride hydrolysis.

Project Description

Objective:

1) Factors which modulate the enzymic activity of lipoprotein lipase (LPL) are of major interest since changes in the extent of hydrolysis of triglyceride and lipoproteins may be associated with hyperlipidemia and atherosclerosis. We have therefore undertaken a systematic evaluation of the effect of human plasma apolipoproteins on the enzymic activity of purified human LPL.

Methods Employed:

Human post heparin plasma containing hepatic lipase (HL) and LPL was obtained from normal subjects following intraveneous injection of sodium heparin (75μ/kg body weight). Blood was collected 15 min. after injection for analysis. LPL was separated from HL by heparin-sepharose affinity chromatography of PHLA. The substrate for enzyme assay was a 3[H]glycerol trioleate-lecithin complex containing bovine serum albumin. Apolipoproteins used for study were purified by standard techniques including gel permeation, ion exchange and affinity chromatography. Of particular importance was the isolation of apoH (β_2-glycoprotein-I) by heparin affinity chromatography of TMU delipidated lymph chylomicrons or triglyceride rich lipoproteins of density < 1.006 g/ml. All apolipoproteins were shown to be homogeneous on SDS gel electrophoresis.

Major Findings:

LPL isolated by heparin affinity chromatography from post heparin plasma was maximally activated 21 ± 4 fold (N=10) by 2.5-10 μg/ml of apoC-II. Concentrations greater than 10 μg/ml were associated with progressively less activation of enzymic activity.

ApoH (β_2-glycoprotein-I) purified from human plasma lipoproteins or thoracic duct lymph also stimulated the enzymic activity of LPL. In experiments (N=5) using LPL isolated from normal subjects (N=2) apoH increased LPL enzymic activity (assayed in the presence of 2.5 μg/ml apoC-II) by 45 ± 17% above central values. ApoH also caused a slight activation of LPL activity in the absence of apoC-II. ApoC-III inhibited the enhanced enzymic activity of LPL produced by apoC-II and apoH + apoC-II. A+ maximal concentrations of apoC-III (40μg/ml), an 86% inhibition of apoC-II, and a 77% reduction of apoH + apoC-II stimulated activity of LPL was observed.

The results from these studies provide evidence for the concept that the enzymic activity of LPL in triglyceride metabolism is modulated by several apolipoproteins including apoH, apoC-II, and apoC-III. These findings may have important implications in elucidating the molecular mechanisms involved in triglyceride metabolism in normal and hypertriglyceridemic subjects.

Significance to Biomedical Research and the Program of the Institute:

The modulation of the enzymic activity of LPL, the major enzyme involved in triglyceride hydrolysis, is of povital importance in our understanding of lipoprotein transport and metabolism. Triglyceride rich lipoproteins accumulate in several clinical disorders of lipoproteins, one of which Type III, hyperlipidemia is associated with atherosclerosis. Other disorders including patients with Types I and V have hypertriglyceredemia often associated with recurrent pancreatitis. The elucidation of a new apolipoprotein, apoH, as a cofactor for the enzyme LPL is of major importance since it is only the second apolipoproteins shown to be associated with increased enzymic activity. These studies will fascilitate our studies which are directed toward the elucidation of the major factors involved in the regulation of triglyceride hydrolysis and lipoprotein catabolism.

Proposed Course:

Detailed analysis of the interaction of apoC-II and apoH on LPL catalysed triglyceride hydrolysis will be continued. The effect of apoH on natural substrates including chylomicrons and VLDL will be investigated. The concentration of apoH in disorders of triglyceride metabolism and hyperlipidemia will be initiated and a systematic research for disorders of apoH metabolism will be undertaken.

Publications:

1. Nakaya, Y., Schaefer, E. J., and Brewer, H. B., Jr.: Activation of Human Post Heparin Lipoprotein Lipase by Apolipoprotein H (β_2-glycoprotein-I). Biochem. Biophys. Res. Comm. (In Press).

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Annual Report of the
Laboratory of Molecular Hematology
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

The Laboratory of Molecular Hematology (LMH) studies the basic molecular mechanisms of gene expression and protein synthesis, specifically using hemoglobin as a model system. LMH is closely associated with the Clinical Hematology Branch (CHB) and collaborates on a number of joint projects. LMH is composed of three segments: the Section of Molecular Genetics, which is primarily concerned with the molecular control of eukaryotic gene expression; the Section of Molecular Cloning, which is primarily concerned with the isolation and characterization of globin and other genes from the genomes of eukaryotic cells; and the Section of Protein Biosynthesis, which is primarily concerned with the mechanism and regulation of hemoglobin synthesis at the translational level.

SECTION OF MOLECULAR GENETICS

The immediate objectives of this section are to: (1) identify, isolate and characterize the regulatory factors and regulatory regions of animal and human DNA which are involved in the control of the expression of the globin genes and (2) develop methods for transferring functional genes into tissue culture cells and intact animals. Information from these programs will be used to study the regulation of globin gene expression in normal and in thalassemic DNA. The long-term goal is to develop means whereby globin gene expression can be corrected in patients with beta thalassemia and other diseases involving abnormal hemoglobin biosynthesis.

Regulatory factors controlling the expression of the globin genes have been identified by a combination of cell biology and molecular biology techniques. Somatic cell hybrids, obtained by fusion of human or animal cells with mouse erythroleukemia (MEL) cells, have been used to provide evidence for positive and negative regulatory factors controlling globin gene expression. In order to purify and characterize these putative regulatory factors, intact-cell and cell-free assays have been established.

Besides somatic cell hybridization, several other methods of gene transfer have been used to insert genes into tissue culture cells. The most successful of these has been the technique of physical microinjection in which a single copy of a specific gene can be injected into the nucleus of a single cell under conditions whereby the injected cell can be grown in culture into a cloned population with or without selective pressure. This technique was successfully used this year (see below) to correct a mouse thymidine kinase (TK) negative L cell by microinjection of a bacterial plasmid containing a TK gene.

The Section has succeeded, during the past year, in demonstrating that: 1) A genetically defective tissue culture cell can be corrected by microinjection of the normal gene. A mixture of two recombinant plasmids was microinjected into mouse TK^- L cells. One plasmid contained a herpes simplex TK gene and the other contained the human beta globin gene. Colonies arising

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from injected cells, incubated in selective medium, were shown to: (a) produce functionally active herpes simplex TK enzyme, (b) replicate the human beta globin gene, and (c) produce human beta globin mRNA sequences at low levels. Thus, the genetic defect (lack of TK activity) was corrected by the microinjected TK gene, and a co-injected human beta globin gene was replicated and weakly expressed.

2) A cell-free transcription assay can be used in purifying transcription initiation factors. The assay, originally developed by Roeder and colleagues for use in locating the promoter region of Ad-2 viral genes, has been set up and modified slightly for use in screening MEL cell fractions for transcriptional initiation factors. A crude extract of MEL cells has been demonstrated to contain transcription initiation factor activity. Fractionation of this crude extract is now underway.

3) The three known strains of alpha thalassemic mice all have a deletion of part or all of their alpha globin gene sequences. In addition, the heterozygous mice have an alpha/beta globin mRNA and an alpha/beta globin chain ratio of around 0.8, rather than 0.5 as expected. Thus, a compensatory mechanism for alleviating the gene deletion mutation appears to exist in these mouse lines.

SECTION OF MOLECULAR CLONING

The objective of this Section is to apply molecular cloning technology to the analysis of genomic DNA sequences involved in the regulation of gene expression in humans and animals.

The Section has succeeded, during the past year, in:
1) Demonstrating that covalently-closed-circular parental and hybrid bacterial plasmids can be rescued from mouse L cells which had been microinjected with parental plasmids. Two recombinant bacterial plasmids were used in the microinjection experiments described above: one containing the TK gene of herpes simplex virus (plasmid χ1), and the second containing a human genomic DNA fragment within which is the human beta globin gene (plasmid pRK1). DNA isolated from one of the microinjected colonies was used in bacterial transformation experiments. pRK1 molecules identical to those originally injected were isolated. In addition, five different classes of recombinant molecules were recovered, all of which appear to contain a common deletion endpoint. Four of the recombinant classes apparently arose from a deletion of segments of pRK1 trimer or dimer molecules, while the fifth recombinant class resulted from recombination between pRK1 and χ1 followed by a deletion event within this recombinant. Extensive reconstitution experiments indicate that these plasmid recombination and deletion events occurred within the mouse cell. Thus, microinjected bacterial plasmids can exist in the non-integrated state in mouse L cells.

2) Subcloning into the plasmids pBR322 and pBR325 various regions of human and mouse genomic DNA from bacteriophage clones. These subclones are being used by both LMH and CHB to facilitate more detailed analysis of globin and TK gene regions. In addition, genomic plasmid clones, together with other plasmid and bacterial clones containing a number of specific globin and TK

gene sequences, have been prepared for use as hybridization probes in a wide range of experiments carried out in both laboratories.

SECTION OF PROTEIN BIOSYNTHESIS

The objectives of this Section are to: (1) determine the mechanisms involved in translational control of protein synthesis; (2) identify and characterize protein and nucleic acid translational components participating in these regulatory mechanisms; and (3) examine the regulation of globin gene expression at the translational level in normal and disease (e.g., thalassemic) states.

Translational regulation of protein biosynthesis by hemin, double-stranded RNA and oxidized glutathione appears to involve changes in the phosphorylation state of eIF-2. It has become apparent, however, that a simple relationship does not exist between alterations of the phosphorylation state of eIF-2 and translational activity. While phosphorylation of eIF-2 appears to be a necessary component of hemin-regulated translational inhibition, it does not appear to be sufficient and other types of covalent modifications (e.g., oxidation/reduction of cysteine residues) may participate in the overall mechanism.

During the past year, the Section has successfully:

1) Purified a specific eIF-2 phosphatase to homogeneity and characterized its activity.

2) Measured the phosphorylation state of eIF-2 in lysate under a variety of physiologic states.

3) Developed reticulocyte lysate translation systems which allow the specific inactivation of major regulatory components such as eIF-2α kinase and eIF-2. This has allowed the study of regulatory mechanisms other than phosphorylation.

4) Demonstrated that oxidation/reduction can regulate translation by mechanisms which do not involve alteration of the eIF-2 phosphorylation state.

5) Demonstrated that the formation of mixed eIF-2:glutathione disulfides can alter site accessibility of eIF-2α kinase.

6) Shown that sodium selenite, a toxic but easily reversible sulfhydryl group reagent, inhibits translational activity by the specific inactivation of eIF-2 by a mechanism involving formation of mixed protein-glutathione disulfides and eIF-2 phosphorylation. The kinetics of inhibition strongly suggest a multistep inactivation of eIF-2 which involves its initial phosphorylation. As a result, a greater understanding of the relationship between eIF-2α kinase, eIF-2α phosphatase, the physicochemical state of eIF-2, and the metabolic state of the cell has been achieved. Detailed investigations of each of these four determinants of the eIF-2 phosphorylation state and their relationship to catalytic eIF-2 recycling are being pursued.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02212-04 MH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Control of Eukaryotic Gene Expression

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. F. Anderson	Chief	MH NHLBI
OTHER:	B. Safer	Medical Officer	MH NHLBI
	E. Tolunay	Visiting Fellow	MH NHLBI
	D. Vembu	Staff Fellow	MH NHLBI
	S. Bernstein	Med. Tech.	MH NHLBI
	Y. Chiang	Chemist	MH NHLBI
	W. Kemper	Chemist	MH NHLBI
	K. Li	Biologist	MH NHLBI
	L. Sanders-Haigh	Med. Tech.	MH NHLBI
	L. Yang	Biologist	MH NHLBI
	N. Young	Expert	CH NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Genetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 7.3	PROFESSIONAL: 2.5	OTHER: 4.8

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to study the regulation of the globin genes at the molecular level. The approach is to identify regulatory factors which influence globin gene expression. Evidence has been obtained for the existence of a positive regulatory factor(s) in induced 2S mouse erythroleukemia cells which can turn on the human fibroblast alpha and beta but not gamma globin genes in MEL x human fibroblast hybrids. Attempts are being made to isolate, purify and characterize regulatory factors using cell-free transcription systems.

PHS-6040
(Rev. 10-76)

Objectives: The objective of this project is to isolate the factors involved in the expression of eukaryotic genes (specifically those for hemoglobin) and to understand how they interact with the regulatory sequences of DNA in order to control gene expression within the intact cell. We have already shown that mouse erythroleukemia (MEL) cells, which are used as a model system for red blood cell differentiation, contain a positive regulatory factor involved in globin gene expression. The procedure is to fractionate these cells into various components which can be individually tested either by physical microinjection into target cells or in cell-free transcription assays for their ability to alter globin gene expression.

Methods:

1) Cells are grown under standard tissue culture conditions. MEL cells are fused by means of inactivated Sendai virus (or polyethylene glycol) to other somatic cells which normally are either capable or incapable of synthesizing hemoglobin. Chromosome and isozyme analyses are done by standard cell biology techniques.

2) Cell hybrids are analyzed for their ability to synthesize globin messenger RNA (mRNA) or globin. Globin mRNA is detected by hybridization with the appropriate complementary DNA (cDNA); the presence of globin is detected by radioactive leucine incorporation followed by either electrophoresis or column chromatography.

Major Findings:

1) The human, beta, and gamma globin genes in the 2S MEL x human fibroblast somatic cell hybrid XX-8 (which was previously shown to produce a globin gene specific positive regulatory factor after induction) have been further characterized. DNase I studies on the chromatin of the uninduced and induced hybrid cells demonstrate that the human gamma globin gene is closed in both states, while the human beta globin gene is closed in the uninduced cell but open in the induced cell. This implies that the positive regulatory factor might act at the level of chromatin conformation. In addition, even though the induced XX-8 cells produced human beta globin mRNA, no human beta globin chains were detected, as shown by carboxymethylcellulose chromatography, acid urea polyacrylamide gels, and antibody displacement reactions. Thus, post-transcriptional regulation of human beta globin synthesis might take place in these cells.

2) Human chromosome (HC) 11 has been selectively retained in the 2S MEL cells by fusing HGPRT$^-$ 2S MEL cells with human fibroblasts containing an X-11 translocation, and then growing the hybrid cells in HAT selective medium. The X portion of the chimeric chromosome contains the HGPRT gene, permitting survival in HAT medium, and the 11 portion contains the beta globin gene as demonstrated by Southern blot analysis. The questions of chromatin structure and post-transcriptional regulation, studied above using XX-8 somatic cell hybrids (in which HC 11 is being randomly lost during cell divisions) are now being studied using a clone of these new hybrid cells: M11X-3.

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3) Transcription initiation factor activity has been detected in crude extracts of 2S MEL cells using the assay published by Roeder and his colleagues for identifying specific initiation on Ad-2 viral templates. Large scale purification procedures are now being set up in order to identify, purify and characterize individual regulatory factors from the 2S MEL cells.

Significance to Biomedical Research and Institute Program:

The molecular control of eukaryotic gene expression remains one of the major questions in biology today. Once it is better understood how a gene in a eukaryotic cell is controlled, this knowledge can be applied to a wide range of human diseases including genetic diseases, viral diseases, cancer, etc.

Proposed Course of Project:

Fractionation of cell components from 2S MEL (and other) cells to identify, purify and characterize individual regulatory factors involved in gene expression. The biological function of the individual factors will be studied both in regards to their ability to interact with DNA and their ability to control the regulation of gene expression.

Publications:

1) Gopalakrishnan, T. V., and Anderson, W. F.: Epigenetic activation of phenylalanine hydroxylase in mouse erythroleukemia cells by the cytoplast of rat hepatoma cells. Proc. Natl. Acad. Sci. USA 76: 3932-3936, 1979.

2) Gopalakrishnan, T. V., and Anderson, W. F.: Mouse erythroleukemia cells. Methods in Enzymology LVIII: 506-511, 1978.

3) Sanders-Haigh, L., Anderson, W. F., and Francke, U.: The human beta globin gene is on the short arm of human chromosome 11. Nature 283: 683-686, 1980.

4) Anderson, W. F.: Control of gene expression. In Reid, C. D. (Ed.): Proceedings of the Conference on Prenatal Diagnosis of Fetal Hemoglobinopathies, NIH Publication Number 80-1529, pp. 227-230, 1980.

5) Anderson, W. F.: Regulation of globin gene expression at the molecular level. Ann. N. Y. Acad. Sci. 344: 262-278, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02213-03 MH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Regulation of Protein Biosynthesis in Cell-Free Systems

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B. Safer	Medical Officer	MH NHLBI
OTHER:	R. Jagus	Visiting Fellow	MH NHLBI
	D. Crouch	Biologist	MH NHLBI
	A. Konieczny	Visiting Fellow	MH NHLBI
	E. Church	Microbiologist	MH NHLBI

COOPERATING UNITS (if any)
P. Cohen, University of Dundee, Dundee, Scotland

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Protein Biosynthesis

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.8	PROFESSIONAL: 2.2	OTHER: 1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Regulation of protein synthesis at the level of mRNA translation is important for: (1) viral shut-off of host protein synthesis, (2) rapid adjustments to changing metabolic conditions, and (3) coordination of heme-globin-biosynthesis during red blood cell maturation. Translational regulation involves changes in the phosphorylation state of the initiation factor eIF-2. We have therefore studied the role of eIF-2 phosphorylation during protein synthesis initiation, and its regulation by the adenylate energy change, the pyridine nucleotide redox state and hemin, in reticulocyte lysate and intact cells. We have identified and characterized four distinct components which regulate the steady state phosphorylation level and activity of the initiator methionyl-$tRNA_f$ binding protein eIF-2, (1) eIF-2α kinase, (2) eIF-2 phosphatase, (3) conformational state of eIF-2 and (4) the oxidation-reduction state of eIF-2. Determination of the eIF-2 pool size, direct chemical measurement of the eIF-2 phosphorylation state, and correlation of these with extent and kinetics of onset of translational inhibition now show that while phosphorylation of eIF-2 does not directly inhibit its activity, phosphorylation is required for additional modifications of eIF-2 which result in conversion from catalytic to stoichiometric utilization of eIF-2.

Objectives: The major goals are to (1) determine the sites and mechanisms of translational control of protein synthesis, (2) to identify, isolate, and characterize the translational and regulatory components involved in such regulation, and (3) to examine the participation of these mechanisms in the regulation of globin gene expression in normal and diseased states. We have purified and characterized a specific eIF-2 phosphatase and are examining the control and specificity of this activity in unfractionated reticulocyte lysate. The relationships between eIF-2, methionyl-tRNA$_f$ binding activity, the oxidation - reduction state of unpaired cysteine residues on eIF-2, and the glucose-6-phosphate dehydrogenase/glutathione reductase system are being investigated. We have developed rapid and sensitive assay procedures to study the regulation of eIF-2α kinase and phosphatase activities in unfractionated systems. Using these techniques, we hope to achieve a quantitative determination of the number of phosphorylation sites and the extent of eIF-2 phosphorylation under a wide variety of physiologic conditions in which the initiation of protein synthesis is regulated at the point of eIF-2-mediated methionyl-tRNA$_f$ binding.

Methods: The phosphorylation state of eIF-2 in unfractionated lysate under different metabolic conditions is determined by two-dimensional polyacrylamide gel electrophoresis or the isolation of eIF-2 using suitable precautions to eliminate eIF-2 kinase and phosphatase activity during the purification procedure. The specific activity of ^{32}P in the adenine and guanine nucleotide pools is maintained constant by substrate level phosphorylation according to the procedure of Glynn and Chappell. Thin layer PEI chromatography is used to measure the specific activities of the nucleotide and phosphate pools. The phosphate content of the eIF-2α and β subunits resolved by two-dimensional polyacrylamide gel electrophoresis is determined by the specific activity of the ATP pool and the size of the eIF-2 pool. The phosphorylation state of eIF-2 isolated from reticulocyte lysate incubated under defined physiologic conditions is determined as alkalai-labile phosphate by the procedure of Ames. Specific pre-initiation complexes formed during the initiation of protein synthesis in normal and inhibited reticulocyte lysate are isolated using sucrose density gradient centrifugation. RNA components are quantitatively identified by nucleotide hybridization techniques. Radiolabeled aminoacyl- and peptidyl-tRNAs are determined as cetyltrimethylammonium bromide precipitate. Protein components are identified by direct or autoradiographic visualization of sucrose gradient fractions following resolution by polyacrylamide gel electrophoresis. Translational components are purified from these specific preinitiation complexes using ion exchange column chromatography, glycerol and sucrose density gradient centrifugation, gel filtration, and affinity column chromatography. Unfractionated messenger-RNA-dependent and gel-filtered reticulocyte lysate preparations, as well as purified translational components, are used in these studies.

Major Findings:

1) The phosphate content of the purified eIF-2α, β and γ subunits has been determined by direct chemical analysis following their purification by CM cellulose chromatography. The phosphate content of eIF-2β is 2 pmol phosphate/pmol subunit. The α and γ subunits of eIF-2 contain less than 0.1 pmol phosphate/pmol subunit. When lysate is incubated in the absence of hemin, the phosphate content of eIF-2β remains unchanged but the phosphate content of

of eIF-2α increases from 0.1 to 0.3 pmol phosphate/pmol subunit. Under all conditions, no phosphate can be detected in eIF-2γ. Using eIF-2 preparations which have previously been totally dephosphorylated by eIF-2 phosphatase, we find that eIF-2β contains two phosphorylation sites which can be phosphorylated by a cAMP-independent eIF-2β kinase. eIF-2α contains a single phosphorylation site which is specifically phosphorylated by the cAMP-independent eIF-2α kinase. Since protein synthesis initiation in hemin-deficient lysate is inhibited by greater than 95%, but the maximum extent of eIF-2α phosphorylation is 30%, it is clear that a simple relationship does not exist between the phosphorylation of eIF-2α and the mechanism of translational inhibition in hemin-deficient lysates.

2) eIF-2 phosphatase has been purified to homogeneity. eIF-2 phosphatase has a native molecular weight of 100,000 daltons and contains one 60,000 and one 38,000 dalton subunit. The activity of eIF-2 phosphatase appears to be regulated primarily by the confirmation of its substrate, eIF-2. In the presence of GTP, eIF-2α is rapidly dephosphorylated. Elevated GDP inhibits both the rate and extent of eIF-2α dephosphorylation. Oxidation of unpaired cysteine residues on the β and γ subunits to form a mixed eIF-2-glutathione disulfide also inhibits the rate and extent of eIF-2α dephosphorylation. Purified eIF-2 phosphatase will dephosphorylate both the α and β subunits of eIF-2. In unfractionated lysate, phosphate present on the β subunit of eIF-2 is not removed under conditions where there is a rapid turnover of phosphate on eIF-2α. The presence of other components in lysate which regulate the specificity of eIF-2 phosphatase is suggested, since the addition of purified eIF-2 phosphatase to unfractionated lysate is accompanied by the loss of its ability to dephosphorylate the β subunit of eIF-2.

3) The mechanism of protein synthesis inhibition which results from the removal of low molecular weight components by gel filtration of lysate has been demonstrated to result from the conversion of eIF-2 utilization from a highly catalytic process to a stoichiometric one. Although biphasic kinetics of inhibition are observed, this inhibition of eIF-2 reutilization does not involve alteration of the phosphorylation state of the eIF-2α subunit. Rather, the oxidation of unpaired cysteine residues on the β and γ subunits of eIF-2 to form mixed eIF-2-glutathione disulfides appears to reduce the capacity of eIF-2 to be reutilized in subsequent rounds of initiation. The specific defect may involve a failure of eIF-2·GDP to dissociate upon release of eIF-2 from the 48S preinitiation complex.

4) The mechanism by which sodium selenite inhibits the initiation of protein synthesis has been determined. Sodium selenite is a unique sulfhydryl group reagent in that, in the presence of excess sulfhydryl group reagents, it spontaneously decomposes to elemental selenium, which is essentially inert. The addition of sodium selenite to unfractionated reticulocyte lysate can directly inactivate eIF-2 since this protein synthesis initiation factor contains sensitive sulfhydryl groups. In addition, the eIF-2α kinase is also activated by sodium selenite. Although the activity of eIF-2 inactivated by selenite can be restored by the addition of NADP-linked substrates which regenerate excess reduced glutathione, the activity of eIF-2α kinase remains elevated. This leads to an increased phosphorylation state of the eIF-2α

subunit. Titration of selenite into reticulocyte lysate can produce a graded extent of eIF-2α phosphorylation. However, despite a six-fold difference in the extent of eIF-2α phosphorylation that can be achieved, the extent of translational inhibition is identical. The correlation that does exist between the extent of eIF-2α phosphorylation is that the time of onset for translational inhibition to occur is decreased the greater the extent of eIF-2α phosphorylation. This may indicate the presence of a multi-step mechanism of inhibition in which the initial phosphorylation of eIF-2α predisposes eIF-2 to undergo a second modification which then is responsible for the inhibition of eIF-2 and protein synthesis initiation.

5) The function of each of the three eIF-2 α, β and γ subunits is being investigated using native preparations of eIF-2 which have been isolated containing only two of the three subunits normally found. Initial studies have demonstrated that the γ subunit of eIF-2 is responsible for binding Met-tRNA$_f$. The α subunit of eIF-2, which undergoes phosphorylation by a specific, hemin-regulated protein kinase, appears to regulate the activity of eIF-2 or its ability to recycle. The β subunit of eIF-2 may be directly involved in the reactivation of eIF-2 and dissociation of GDP following its release from the 48S preinitiation complex.

Significance to Biomedical Research and Institute Program:

Although regulation at the level of transcription is currently thought to be the primary mechanism for regulating the flow of genetic information, modulation of protein synthesis has recently assumed increased importance. Translational regulation has been identified as a major feature of host-virus interaction. Regulation of protein synthesis by hemin was once thought to be a highly specialized means of coordinating globin chain and hemin synthesis in reticulocytes; it now appears, however, to be a widespread mechanism for post-transcriptional modulation of gene expression involving a cascade of highly specific protein kinases and other covalent modifiers. Final control of gene expression during cell differentiation may also be regulated by interaction of translational components with messenger RNA. It is essential, therefore, to understand the basic mechanisms involved in these processes to be able to control gene expression in the cell.

Proposed Course of the Project:

The specific kinase and phosphatase which regulate the phosphorylation state of eIF-2 will be used to obtain preparations of eIF-2 in a defined phosphorylation state. Peptides containing the phosphorylation sites will be isolated using high pressure liquid chromatography and sequenced. The redox system present in unfractionated reticulocyte lysate which influence the formation of mixed eIF-2-glutathione disulfides will also be examined. The overall goal of both of these approaches will be to understand the mechanism which normally allows catalytic recycling of eIF-2, and the defect(s) which occur during translational regulatory mechanisms which convert catalytic utilization of eIF-2 to stoichiometric utilization and translational inhibition. We have also initiated studies leading to the establishment of an in vitro transcription system which will be used to examine the mechanisms involved in the regulation of genetic expression at both the transcriptional and translational levels.

Publications:

1. Jagus, R., and Safer, B.: Quantitation and localization of globin messenger RNA in reticulocyte lysate. J. Biol. Chem. 254: 6865-6868, 1979.

2. Peterson, D. T., Safer, B., and Merrick, W. C.: Role of eIF-5 in the formation of 80S initiation complexes. J. Biol. Chem. 254: 7730-7735, 1979.

3. Safer, B., Kemper, W., and Jagus, R.: The use of $[^{14}C]$eIF-2 to measure the endogenous pool size of eIF-2 in rabbit reticulocyte lysate. J. Biol. Chem. 254: 8091-8094, 1979.

4. Lloyd, M. A., Osborne, J. C., Safer, B., Huff, G. P., and Merrick, W. C.: Characteristics of eukaryotic initiation factor 2 and its subunits. J. Biol. Chem. 255: 1189-1193, 1980.

5. Safer, B., Jagus, R., Crouch, D., and Kemper, W.: Regulation of eIF-2 activity in reticulocyte lysate. In Gordon, J. and Thomas G. (Eds.): Protein Phosphorylation and Bioregulation. Basle, A. Karger AG, 1980, in press.

6. Crouch, D., and Safer, B.: Purification and properties of eIF-2 phosphatase. J. Biol. Chem., in press, 1980.

7. Safer, B., Jagus, R., and Crouch, D.: Indirect inactivation of eIF-2 in reticulocyte lysate by selenite. J. Biol. Chem., in press, 1980.

8. Jagus, R., Anderson, W. F., and Safer, B.: Initiation of mammalian protein synthesis. Prog. Biophys. Mol. Biol., in press, 1980.

9. Stewart, A. A., Crouch, D., Cohen, P., and Safer, B.: Classification of an eIF-2 phosphatase as a type-2 protein phosphatase. FEBS Lett., in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02214-03 MH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Molecular Cloning of Eukaryotic Globin Gene Sequences

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Kretschmer	Staff Fellow	MH NHLBI
OTHER:	W. F. Anderson	Chief	MH NHLBI
	H. Coon	Biologist	MH NHLBI
	A. Bowman	Microbiologist	MH NHLBI
	E. Schmader	Chemist	MH NHLBI
	A. Nienhuis	Chief	CH NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Cloning

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.3	0.7	1.6

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to apply recombinant DNA and cloning technology to the isolation and analysis of genomic DNA sequences involved in the regulation of globin gene expression in humans and animals. Fragments of DNA relating to globin and thymidine kinase gene expression in humans and/or animals are isolated from genomic clones (constructed in this and other laboratories) and subcloned into plasmids for use in studies on eukaryotic gene expression. A prokaryote-eukaryote-prokaryote DNA transfer and recovery system has been developed which should be useful in future experiments on the regulation of gene expression in eukaryotes.

Objectives: The objective of this project is to establish and apply plasmid, bacteriophage and cosmid recombinant DNA cloning systems for the isolation and analysis of human and animal genomic DNA sequences involving the regulation of globin and thymidine kinase (TK) gene expression.

Methods:

1) Molecular cloning is by use of the in vitro packaging system of bacteriophage lambda. The in vitro packaging system requires three components. The first component involves preparation of three bacterial extracts: protein A, freeze-thaw lysate, and sonic extract, which provide between them all the proteins needed to form bacteriophage particles in vitro. The second component is the preparation of the vector DNA, which in our case is usually the outer Eco RI "arms" of the vector, charon 4A. The third component is the isolation and purification of 15-20 kb segments of genomic DNA containing Eco RI ends. The first step in the cloning procedure is to ligate the charon 4A arms with the genomic DNA such that long linear concatemers of DNA result. This in vitro recombinant DNA can then be efficiently packaged into bacteriophage particles using the bacterial extracts of component 1 (see above), resulting in a cloned library of one million segments of genomic DNA. This library is then screened with radioactively labeled probe by well-established plaque hybridization methods.

2) Subcloning smaller segments of these clones is done into bacterial plasmid vectors (such as pBR313, pBR322, pBR325). The plasmid vector is digested with a suitable restriction endonuclease, mixed and ligated with the fragment to be subcloned, and transformed into bacterial cells. Those colonies containing the required plasmid clone can be determined first by antibiotic resistant tests and secondly, by examination by restriction endonuclease and Southern blot analysis of their plasmid DNA on agarose gels.

Major Findings:

1) A prokaryote-eukaryote-prokaryote DNA transfer and recovery system was developed in which a human beta globin gene was inserted into a bacterial plasmid, the plasmid containing the globin gene was microinjected into a mouse L cell where it multiplied and underwent recombinant events, and the same plasmid was then recovered (in the original as well as in mutant forms) by transformation of susceptible bacteria with DNA from the microinjected mouse cells. In an accompanying project report (Z01 HL 02216-01 MH), the isolation of TK^+ human beta globin gene containing colonies following co-microinjection of mouse TK^- L cell nuclei with two recombinant bacterial plasmids was described. The two recombinant plasmids were: (a) $\chi 1$ (a pBR322 plasmid containing the TK gene of herpes simplex virus) and (b) pRK1 (pBR322 containing a human genomic DNA fragment within which is the human beta globin gene). DNA isolated from one of the microinjected clones was used in bacterial transformation experiments with a selection for tetracycline-resistant colonies (that is, for cells containing pRK1). A total of forty-three tetracycline-resistant colonies were isolated, thirty of which contained pRK1 molecules identical to those originally injected. The remaining thirteen colonies contained plasmids that were grouped into five

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different classes of recombinant molecules. All five of these recombinant classes appear to contain a common deletion endpoint occurring at a similar region of the pBR322 segment of pRK1. Four of the recombinant classes apparently arose from a deletion of segments of pRK1 trimer or dimer molecules, while the fifth recombinant class resulted from recombination between pRK1 and $\chi 1$ followed by a deletion event within this recombinant. Extensive reconstitution experiments indicate that these plasmid recombination and deletion events occurred within the mouse cell. Recovery of intact pRK1 molecules from the mouse DNA (at a level of 0.5 extra-chromosomal plasmids per mouse cell) demonstrate that microinjected bacterial plasmids can exist in the non-integrated state in mouse L cells. We have, therefore, established a prokaryote-eukaryote-prokaryote DNA transfer and recovery system which should be useful in future experiments on the regulation of gene expression in eukaryotes.

2) A number of human, sheep, and mouse genomic DNA fragments containing globin and collagen genes have been subcloned into a variety of plasmids. For collaborations on sheep globin genes with the Clinical Hematology Branch, see their Project Reports. For collaborations on collagen genes with the Pulmonary Branch, see their Project Reports.

Significance to Biomedical Research and Institute Program:

The technique of recombinant DNA technology is a new and extraordinarily powerful technique for manipulating genetic material. Specific genes can be isolated, purified and characterized. Increased knowledge of the structural organization and sequence of normal and mutated human genes should provide further understanding into the mechanisms of genetic diseases.

Proposed Course of the Project:

The prokaryote-eukaryote-prokaryote DNA transfer and recovery system will be used to study the feasibility of transferring functional genes into mammalian cells. Isolation and characterization of genomic genes and gene fragments will continue.

Publications:

1) Kretschmer, P. J., and Cohen, S. N.: Effect of temperature on the translocation frequency of the Tn3 element. J. Bacteriol. 139: 515-519, 1979.

2) Kretschmer, P. J., Bowman, A. H., Sanders-Haigh, L., Killos, L., and Anderson, W. F.: Recovery and characterization of recombinant bacterial plasmids from microinjected mouse L cells. Cell, in press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02215-02 MH

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Etiology of Three Mouse Alpha-Thalassemias

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. B. Whitney III	Expert	LMH NHLBI
OTHER:	J. Martinell	Staff Fellow	LMH NHLBI
	W. F. Anderson	Chief	LMH NHLBI

COOPERATING UNITS (if any)
R. A. Popp and L. C. Skow, Oak Ridge National Laboratory, Oak Ridge, TN;
M. Potter, Laboratory of Cell Biology, NCI

LAB/BRANCH
Laboratory of Molecular Hematology

SECTION
Section of Molecular Genetics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.1	2.1	0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Three induced mutations of the mouse are true alpha-thalassemias which map to or near the Hba alpha globin structural gene locus. Although two were independently induced with x-rays and the third with triethylenemelamine, all appear to have a similar cause: an incomplete deletion of the alpha gene complex. In each, the adult globin genes were previously shown to be fully inactive: we now have proven that at least one (probably both) of the two adult alpha genes per chromosome is actually deleted. Other parts of the alpha locus complex are still present but these apparently are not being expressed as proteins at detectable levels.

Apparently normal variations at the globin gene DNA level have also been found among inbred and exotic mouse stocks.

Messenger RNA levels in the thalassemia heterozygotes are around 80% of normal, implying effective compensation for the loss of one gene set.

PHS-6040
(Rev. 10-76)

Objectives: The objective of this work is to characterize fully the nature of the defect which obliterates the expression of alpha globin genes in three induced mouse alpha-thalassemias.

Methods:

(1) DNA prepared from genetically-characterized normal and heterozygous alpha-thalassemic mice is digested with restriction endonuclease Eco RI, then transferred to nitrocellulose membrane filters following 0.8% agarose gel electrophoresis. Alpha (or beta) globin genes or fragments are detected by hybridization with probes made by nick-translation of parts of the globin DNA clones, Charon 3A-117 alpha (or 3A-4beta), Mαl, or Ch3AMm30.5.

(2) Total reticulocyte RNA, erythroid cell nuclear or cytoplasmic RNA, or genomic DNA is analyzed by liquid hybridization with ^{32}P-labeled single-stranded alpha-or beta-globin cDNA probes.

(3) Hemoglobins and globins are analyzed by slab polyacrylamide gel isoelectric focusing, electrophoresis, and ion-exchange chromatography.

(4) A simple osmotic resistance test is used to detect thalassemic mice for breeding.

Major Findings:

(1) Genetic studies indicate that each of the three thalassemias maps to mouse chromosome 11 near the wa-2 locus, the normal location of the Hba adult alpha chain locus. Lower recombination than expected between wa-2 and the 352HB mutant locus is consistent with the mutation being a small deletion in chromosome 11.

(2) By Southern blot transfer methods, four normal varieties of alpha globin gene DNA patterns have been found. DNA patterns from heterozygotes with thalassemia and a normal variant DNA chromosome show that one particular 3 kb DNA region is deleted in each of the three thalassemias. This deletion includes one entire adult alpha gene which normally lies on an 11 kb Eco RI segment of genomic DNA. There is a quantitative indication that the comparable 3 kb region including the other adult alpha globin gene is also deleted. Nonetheless, two other fragments containing alpha-like sequences are not deleted in any of the thalassemias. This indicates that one deletion breakpoint of each mutation lies within the alpha gene cluster, between the adult genes and the remaining alpha-like genes. The precise locations of these breakpoints within the clusters remain to be determined. Locations of these breakpoints both relative to the inactivated α locus and among the three thalassemics will be compared.

(3) Total reticulocyte RNAs from each type of thalassemic mouse have been analyzed by liquid hybridization with double-labeled single-stranded alpha- and beta-globin cDNA probes. The results are consistent with our earlier protein synthesis studies which showed surprisingly slight reduction of alpha relative to the beta synthesis in comparison to normal controls analyzed in

parallel.

(4) Mouse stocks have been created which are homozygous for four of our five newly-discovered Hba genotypes. DNA samples have been or will be analyzed for qualitative variations. Blood samples have been or are being collected for alpha globin sequence analysis by Dr. R. A. Popp at the Oak Ridge National Laboratory.

Significance to Biomedical Research and Institute Program: These thalassemias are the first mammalian mutations induced *in vivo* to be analyzed at the genomic DNA level. These thalassemic mice are the best models currently available for a human molecular disease and may be invaluable in the development of methods for the direct therapy of human genetic diseases.

Proposed Course of the Project:

The nucleic acid analysis aspect of the project is essentially completed. Dr. Whitney will continue more detailed studies of the gene deletions at the Medical College of Georgia. Ms. L. Killos will attempt to microinject cloned alpha globin genes into homozygous thalassemic embryos in an attempt to correct the mutation.

Publications:

1. Popp, R. A., Skow, L. C., and Whitney, J. B. III: Expression of embryonic hemoglobin genes in alpha-thalassemic and in beta-duplication mice. *Ann. N. Y. Acad. Sci.* *344*: 280-283, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02216-01 MH

PERIOD COVERED October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Correction of Genetic Defects by Gene Transfer

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	W. F. Anderson	Chief	
OTHER:	M. Huberman	Research Associate	MH NHLBI
	B. Safer	Medical Officer	MH NHLBI
	J. Shapiro	Staff Fellow	MH NHLBI
	J. DiPietro	Biologist	MH NHLBI
	L. Killos	Biol. Lab. Tech.	MH NHLBI
	L. Sanders-Haigh	Med. Tech.	MH NHLBI
	A. Nienhuis	Chief	CH NHLBI

COOPERATING UNITS (if any)

E. G. Diacumakos, Ph.D., Rockefeller University, New York, New York

LAB/BRANCH

Laboratory of Molecular Hematology

SECTION

Section of Molecular Genetics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS: 3.4	PROFESSIONAL: 1.3	OTHER: 2.1

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methods are being sought for transferring functional genes into mammalian tissue culture cells and ultimately into intact animals. A primary technique is physical microinjection of specific genes into the nucleus of individual tissue culture cells. A genetic defect, lack of thymidine kinase (TK) activity, was corrected in mouse TK⁻ L cells by microinjecting a herpes simplex TK gene. In addition, a co-injected human beta globin gene was replicated and expressed at a low level in these cells.

PHS-6040
(Rev. 10-76)

Objectives: The objective of this project is to develop methods for transferring functional genes into mammalian tissue culture cells and into intact animals. Ultimately, the techniques would be used for attempting to correct genetic diseases in humans.

Methods:

1) Tissue culture cells are grown under standard tissue culture conditions.

2) Plasmids containing specific functional genes are made as described in the accompanying project report (Z01 HL 02214-03 MH).

3) Physical microinjection into specific regions of intact mammalian cells is carried out by using ultra-thin micropipettes under 2,000-fold phase optics according to the procedure of Diacumakos (Diacumakos, E.G., Methods in Cell Biology 7: 287-311, 1973). Single cell clones are grown to 10^8-10^9 cells for isolation of DNA and RNA for analysis by restriction endonuclease and Southern blot analysis, by mRNA-cDNA liquid hybridization, or by Northern blot analysis.

Major Findings:

1) A mixture of two recombinant plasmids was microinjected into mouse TK^- fibroblasts (L cells). One plasmid contained the herpes simplex type 1 TK gene and the other contained the human beta globin gene. Approximately one or twenty copies of each plasmid were injected. Seven fibroblast colonies arising from injected cells and incubated in hypoxanthine-aminopterin-thymidine (HAT) medium were analyzed. These microinjected cells were shown to: (a) produce functionally active herpes simplex type 1 TK enzyme, (b) replicate the human beta globin gene, and (c) produce human beta globin mRNA sequences at low levels. Thus, the genetic defect (lack of TK activity) was corrected by the microinjected TK gene, and a co-injected human beta globin gene was replicated and weakly expressed.

2) The TK and human beta globin genes were inserted into the same plasmid (pBR325). This hybrid plasmid (called pPK39) containing two functional genes was microinjected into mouse TK^- L cells. Clones of HAT resistant cells were grown and shown to contain human beta globin gene sequences by restriction endonuclease and Southern blot analysis.

Significance to Biomedical Research and Institute Program:

The long-term aim of much of the work in molecular genetics is to develop techniques for treating or curing human genetic defects. This project utilizes recombinant DNA technology and cell biology techniques to try to accomplish this goal.

Proposed Course of the Project:

Attempts are continuing to microinject globin genes into erythroid cells (viz. mouse erythroleukemia cells) for examining the globin gene in an erythroid environment. In addition, the injection of functional genes into mouse embryos to attempt to establish gene transfer in intact animals is planned.

Publications:

1) Anderson, W. F., Killos, L., Sanders-Haigh, L., Kretschmer, P. J., and Diacumakos, E. G.: Replication and expression of thymidine kinase and human globin genes microinjected into mouse fibroblasts. Proc. Natl. Acad. Sci. USA, in press, 1980.

2) Anderson, W. F., Killos, L., Sanders-Haigh, L., Kretschmer, P., Diacumakos, E., Nienhuis, A., Willing, M., and Vembu, D.: Regulation of human globin gene expression after gene transfer. In Sigler, P.B. (Ed.): Molecular Basis of Mutant Hemoglobin Dysfunction (University of Chicago Symposia on Sickle Cell Anemia), Vol. 1. New York, North-Holland Publishing Co., in press, 1980.

3) Diacumakos, E. G., Killos, L., Lee, L., and Anderson, W. F.: Induction of mouse erythroleukemia cells by microinjection of inducing compound. Expt. Cell Res., in press, 1980.

Annual Report of the
Section on Laboratory Animal Medicine and Surgery
Office of the Director of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1979 to September 30, 1980

The Section functions primarily in a support role to all laboratories of IR providing care for many species of animals, technical assistance in preparation and maintenance of animal models for various experimental regimens, and the development of animal resources not otherwise available.

Maintenance of various rodent and aquatic species has been accomplished in designated areas in close proximity to IR laboratories in Buildings 3, 10, and 36. Large animal species are maintained in Buildings 3, 28, the NIHAC, and at Luray, Virginia. Postoperative intensive care and treatment of surgery patients is completed in Buildings 3, 14-E, and 28.

The animal surgery laboratory located in Building 14-E supports the Clinical Hematology Branch, Laboratory of Experimental Atherosclerosis, Hypertension-Endocrine Branch, Laboratory of Technical Development, the Surgery Branch, and the Pulmonary Branch in preparation of experimental animal models, completing cardiovascular studies and in collecting various biological specimens. The laboratory operates an x-ray catheterization suite, clinical chemistry laboratory, sterile operating suites, and special study suites required to meet requirements.

The NHLBI Sheep Colony has again continued successful year-round breeding of laboratory sheep. Approximately 500 animals were delivered to laboratories meeting requirements of gestation stages from 22-140 days and various age and size lambs, young adults, and aged sheep.

Laboratory studies have been underway to define hemodynamic parameters and tissue morphology resultant of Newfoundland dog left ventricular hypertrophy due to infracoronary left ventricle outflow tract obstruction. Disease processes will be characterized to determine usefulness of this unique animal model for future laboratory study.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-03401-04-LAMS

PERIOD COVERED
10/1/79 through 9/30/80

TITLE OF PROJECT (80 characters or less)

Newfoundland Breeding Colony

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. E. Pierce	Chief, SLAMS, NHLBI
	D. K. Buckhold	SLAMS, NHLBI
	J. F. Harwell, Jr.	SLAMS, NHLBI
Other:	M. Jones	SB, NHLBI
	D. E. Wildt	VRB, DRS
	K. D. Wright	VRB, DRS

COOPERATING UNITS (if any)
Veterinary Resources Branch, DRS

LAB/BRANCH
Office of the Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4.0	3.0	1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Newfoundland Breeding Colony has been developed as a source of laboratory dogs affected with left ventricular hypertrophy (LVH) due to infracoronary left ventricle outflow tract obstruction and other spontaneously occurring heart defects. More than 100 dogs have been reared, all having some form of hereditary subaortic stenosis (SAS), with one family demonstrating pulmonic valve stenosis and SAS.

Cardiac catheterization of more than 100 dogs has demonstrated varying peak systolic pressure gradients between left ventricle and aorta from < 5 to > 150 mmHg. Laboratory studies are presently underway to define hemodynamic parameters and tissue morphology resultant of Newfoundland discrete SAS.

Numbers of animals have been used for regional myocardial blood flow studies of the hypertrophied ventricle and evaluation of corrective surgery techniques using apico-aorta prosthetic devices.

The Newfoundland Breeding Colony was maintained by Flow Laboratories, Inc., at Dublin, Virginia supported by NIH 263-78-D-0253. Twenty-five dogs remain and are located in Building 28.

Varying degrees of severity of LVH existed in dogs studied from the colony and gross and microscopic changes of heart tissue existed similar to those occurring in human patients with similar cardiac abnormalities.

Availability of a naturally occurring animal model for study of LVH resultant of infracoronary LV outflow tract obstruction is important because technical difficulties have not been satisfactorily overcome in attempts to produce such a defect in normal animals.

Discrete subaortic stenosis has been studied in Newfoundland dogs at the School of Veterinary Medicine, University of Pennsylvania where initial breeding experiments suggested that it is inherited and either a polygenic or an autosomal dominant trait with modifiers.

More than 100 cardiac catheterizations have demonstrated peak systolic blood pressure gradients between left ventricle and aorta (LV-aorta PG) range from < 5 mmHg to > 150 mmHg.

All hearts grossly examined except those less than 60 days old have demonstrated a fibrous membrane located 3-8 mm below the sinus of valsalva. The discrete narrowing varies in different hearts. Severely effected LV outflow tracts have circumferential discrete narrowing and extend transversely from the anterior mitral leaflets at the level of its attachment to the atrial septum and the outflow surface of the leaflet. Some lesions in the more severely affected dogs appear to be similar to a condition described in human heart disease known as tunnel subaortic stenosis.

Varying degrees of concentric left ventricular hypertrophy occurs: (1) in all dogs > 3 years old; (2) in all age groups with LV-aorta PG > 80 mmHg; (3) Severe hypertrophy is present in dogs several years old with LV-aorta PG > 125 mmHg.

Histologic and ultrastructural studies showed that the subaortic fibrous "ring" present in Newfoundland dogs with discrete SAS lacked the layered structure of normal endocardium in left ventricular outflow tract. The fibrous ring tissue was characterized by the presence of large uni- and multinucleated, rounded connective tissue cells that resembled chondrocytes in several respects. The cells were surrounded by connective tissue rich in acid mucophysaccharides, small but cross-banded collagen fibrils and small, poorly developed elastic fibers. The unusual differentiation of cellular and extracellular components of connective tissue in subaortic fibrous rings in dogs with discrete SAS clearly differs from that seen in humans with discrete SAS.

Morphologic studies of small intramural coronary arteries revealed a > 50% incidence of narrowing of these vessels by proliferation of smooth muscle cells (SMC) in intima and by fibrosis involving intima, media, and advantitia. The

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most striking ultrastructural abnormality involves intimal elastic tissue which forms numerous small, scattered fibrils (>1μ in diameter) rather than discrete laminae separating layers of SMC. Collagen fibers between SMC are increased in number but are morphologically normal. The changes described resemble those in humans with hypertrophic cardiomyopathy and with discrete ring or tunnel types of SAS.

Dogs with pressure gradients above 100 mmHg are susceptible to syncope, "Sudden Death Syndrome" or atrial fibrillation and eventual death from heart failure.

A breeding program has been set up with VRB, DRS to produce animals for hemodynamic and morphologic studies between the age of newborn and four months.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Murra, W.F.T., Ferrans, V.J., Pierce, J.E. and Roberts, W.C.: Discrete Subaortic Stenosis in Newfoundland Dogs: Association of Infective Endocarditis. Am. J. of Cardiol. 41: 746-754, 1978.

Murra, W.F.T., Ferrans, V.J., Pierce, J.E. and Roberts, W.C.: Ultrastructure of the Fibrous Subaortic "Ring" in Dogs with Discrete Subaortic Stenosis. Lab. Invest. 39: 471-482, 1978.

789

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-03402-04-LAMS

PERIOD COVERED
10/1/79 through 9/30/80

TITLE OF PROJECT (80 characters or less)

NHLBI Laboratory Sheep Colony

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. E. Pierce — Chief, SLAMS, OD, NHLBI

Other: J. F. Harwell, Jr. — SLAMS, OD, NHLBI

COOPERATING UNITS (if any) (1) Laboratory of Biomedical Sciences, IRP, NICHHD, (2) NINCDS, IRP, LPP, (3) DOD - USAMRIID, Animal Assessment Div., Ft. Detrick, MD, (4) ARB, DVR, FDA, (5) ACS, VRB, DRS.

LAB/BRANCH
Office of Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	0.8	0.2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The laboratory Sheep Colony is an NIH animal resource providing varied age animals that meet specific year-round requirements of the Clinical Hematology Branch, Laboratory of Technical Development, Pulmonary Branch, and Surgery Branch, DIR, NHLBI; the Laboratory of Biomedical Sciences, IRP, NICHHD; LPP, IRP, NINCDS. Maintenance regimens in use have resulted in successful year-round breeding and production of varied age sheep.

Practices that have contributed to reduction of undesired seasonal variables include: (1) continuous prophylactic immunization of all age animal groups; (2) accurate pregnancy diagnosis during first trimester using Doppler ultrasound; (3) monitoring of animal health using various diagnostic laboratory techniques; and (4) many husbandry techniques unique to this colony. Such practices have been cost prohibitive in commercial sheep flocks that result in inconsistent availability and existence of varied states of health in animals delivered for laboratory use.

PHS-6040
(Rev. 10-76)

Project Description: Z01-HL-03402-04-LAMS

The breeding colony continues as a source of sheep with A, AB, and B type hemoglobin for the CHB and meets other supportive laboratory program requirements of young lambs and pregnant ewes as required. From 700 to 950 varied age sheep exist in the colony year-round. Approximately 500 animals were delivered to NIH and other facilities for laboratory use during the report period.

The contractor has been responsible for developing and updating husbandry techniques as instructed by the project officer that allow optimal conditions for natural year-round breeding at the contract site. The project officer has been responsible for monitoring techniques and updating guidelines followed by the contractor to meet NIH laboratory requirements along with animal health regimens (sheep property of NIH) that allow minimal susceptibility of all age groups to common sheep diseases.

Immunization protocols direct contract personnel to administer specific ovine and bovine bacterins, and tetanus toxoid to lambs with biweekly boosters of each by the fifth week of age. This practice has essentially eliminated enterotoxemia and more than 90% of chronic pneumonia previously experienced. Repeated administration of indicated biologicals is carried out in all age groups at designated periods of development and production.

The necessity of an accurate method of pregnancy diagnosis during early gestation was determined during initial development of the colony. Natural seasonal and environmental conditions effect the weekly conception rate varying from 0-100%. Lindahl's technique using Doppler ultrasound with rectal examination has been adequate. It allows accurate diagnosis of up to 100% of pregnant sheep from 21-35 days gestation. Negative animals must be re-examined at least twice to verify non-pregnancy. Examinations are performed weekly by contract personnel with more than 1,500 examinations completed per year to detect approximately 500 pregnant ewes conceived over a 52-week period.

Laboratory tests are continuously performed to monitor flock health. Contract personnel monitor internal parasite infestation by random fecal sampling from various animal groups with examination using direct and flotation methods. CBC and blood chemistry profiles are performed on animals delivered to the laboratory to monitor health and nutrition states. Microbiological and serological screening for detection of suspected disease entities is carried out when indicated.

This project will continue as IR and other NIH programs have sufficient requirements that justify the continued support of this laboratory sheep resource. Production goals and total numbers of animals maintained will be varied as required by changing demands of laboratories.

Contract Information:

Contract Number:	263-80-C-0007 - approximately $210,000 - 10/1/79 - 9/30/80
Contract Site:	White House Farms, Inc. Box 403-E Luray, Virginia 22835
PI:	Max Foltz, Contractor Rick Miller, Colony Manager
Total Manyears:	8.0
Professional:	2.0
Other:	6.0

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01-HL-03403-03-LAMS

PERIOD COVERED
10/1/79 through 9/30/80

TITLE OF PROJECT (80 characters or less)
Indirect Blood Pressure Measurements in Laboratory Animals Using Oscillometry.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

J. E. Pierce	Chief, SLAMS, OD, NHLBI
E. Walker	BEI, DRS

COOPERATING UNITS (if any)

E. Walker	BEI, DRS

LAB/BRANCH
Office of Director of Intramural Research

SECTION
Section on Laboratory Animal Medicine and Surgery

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.5	1.0	0.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) A technique for obtaining indirect blood pressure measurements in laboratory animals currently being evaluated, oscillometry, monitors the oscillations in cuff pressure with a pressure transducer as the cuff is deflated. During deflation from super systolic levels, the first increase in oscillation observed is designated as systolic blood pressure.

Mean arterial measurements obtained on anesthetized dogs with direct pressure measurements available were consistently within 1-3% of direct pressure.

Measurements obtained on awake dogs were not as consistent but corresponding values were obtained in both forelegs over a 15-minute examination period within 5% of each other. (Direct measurements not simultaneously available).

The technique will be further evaluated using a commercially available unit and a laboratory designated oscillometer which provides raw oscillation data to assist in determining optimal cuff sizes to be used and identify other variables that effect obtaining accurate blood pressure measurements in laboratory animals.

A technique for obtaining accurate indirect blood pressure measurements in laboratory animals, particularly dogs and miniature swine has been continuously requested by investigators of the Laboratory of Experimental Atherosclerosis, Hypertension-Endocrine Branch, and Surgery Branch, IR.

A technique using Doppler ultrasound with transducer secured to an inflatable cuff has proved unsatisfactory. Inconsistent blood pressure values were obtained in awake and anesthetized animals locating the cuff at various sites on the forelimb, rear leg, and tail as reported successful by other groups.

Because of the unreliability of traditional techniques, we have been investigating a technique called oscillometry. Oscillometry is a method of measuring blood pressure by analyzing the pulse pattern of the cuff pressure oscillations.

In practice, a cuff is placed around a limb and inflated to a pressure above systolic and then slowly deflated. While the cuff is being deflated the amplitude of oscillation in cuff pressure, produced by the arterial pulse beneath the cuff, is monitored. Systolic pressure is indicated by the first significant increase in oscillation amplitude. Diastolic pressure is indicated as the lowest cuff pressure at the point of maximum oscillation in cuff pressure. Currently, most investigators using this technique take the point of maximum oscillation to indicate mean arterial pressure.

The results of 19 dog experiments and many laboratory simulations indicate that oscillometry measures systolic and diastolic pressures but not the mean arterial pressure. The mean arterial pressure can be incorrectly indicated when there are unknown errors present in the technique.

ANNUAL REPORT OF THE
SECTION ON THEORETICAL BIOPHYSICS
OFFICE OF THE DIRECTOR OF INTRAMURAL RESEARCH
NATIONAL HEART, LUNG, & BLOOD INSTITUTE
October 1, 1979 through September 30, 1980

The primary interest of the Section on Theoretical Biophysics is the theory of transport processes in biological systems, with particular reference to problems in cardiovascular, renal, and membrane physiology. The section is concerned both with the formulation of theoretical models and with the development of mathematical and computational methods for their analysis. Currently much of the research in the section centers on the mechanism of urine formation in the mammalian kidney and on theoretical aspects of solute and water transport in epithelial structures.

During the past year, areas of work have included: (1) The thermodynamic and kinetic analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, (3) the development of analytical solution of kidney models, (4) the development and theoretical analysis of numerical methods, (5) the computer simulation of renal function, (6) the computer simulation of solute and water transport in simple epithelia, and (7) the development of analytical models of epithelia.

Specifically: During the past year in work on the theoretical analysis of renal function, we have derived a relation between volume flow, concentration, and tubal solute and water permeabilities and reflection coefficients for a flow tube exchanging a single neutral solute and water with the surrounding interstitium. When this relation is combined with the normalized mass balance equation for a central core model of the renal medulla, we obtain a transcendental equation in the concentration ratio of the system and the permeabilities and reflection coefficients of the descending limb of Henle and the collecting duct. In general one must solve this equation numerically, but for some special cases it can be solved analytically. Depending on the choice of parameters this equation may have no roots, a single positive root or more than one positive root for r. Thus, in some models we find that for a given set of parameters and boundary values the concentration profile may be nonunique.

This analytic result has been confirmed by extensive numerical experiments. Numerical techniques have been developed (i) to obtain all solutions to a model for a given set of parameters and (ii) to follow the solution surface as a function of any one of the model's parameters. The models investigated so far have had either no solution, one, two, or three solutions. It is yet not established whether these multiple steady states are modelling artifacts or have physiological significance. Three test criteria have so far been identified: (i) The time stability of solutions (ii) their thermodynamic energy requirement, and (iii) the shape of the solution surface and the ability of the corresponding physiological system to proceed from one state to another on this surface. By using these criteria, it has usually been possible to identify many solutions as physiologically impossible or improbable, but the possibility that under appropriate circumstances multiple steady states exist remains open.

During the past year we have also analyzed mass balance in a single stage of a multistage model of the renal medulla. In earlier work it was shown that the mass balance equation for a variety of models of the renal medulla can be cast into the normalized form

$$r = 1/[1 - f_T(1 - f_U)(1 - f_W)],$$

where r is the ratio of total osmolality at the papilla (assumed approximately the same in all structures) to plasma osmolality, f_T is fractional solute transport out of ascending Henle's limb. f_U is the fractional urine flow, and f_W, the fractional dissipation in the vascular exchanger, is a measure of the solute that is returned to the systemic circulation by ascending vasa recta unaccompanied by its isotonic equivalent of water. This form of the mass balance equation has proved very useful in understanding the qualitative behavior of medullary models and in checking detailed calculations. With suitable interpretations of f_T, f_U, and f_W, the equation applies to multinephron models as well as single nephron models of the medulla, but only gives an overall concentration ratio. In our current work we have derived a form of the equation that is applicable to a single stage of a multistage model: Applied recursively this form permits a much finer analysis of the behavior of multistage systems.

Besides the exploration of multiple solutions, work on the simulation of renal function included the development of a benchmark problem for investigators to use to validate their numerics. This is a six tube vasa recta model that has now been solved with a number of numerical schemes.

In collaboration with R. Tewarson (SUNY), research on the numerical solution of nonlinear ordinary and partial differential equations has continued. In particular a higher order integration scheme that does not increase the connectivity of the equations has been developed and is being tested on a central core model.

In collaboration with B. Kellogg and J. Garner work has continued on the qualitative analysis of equations describing kidney models. A study has been made of a number of problems, each dealing with a different set of differential equations and boundary conditions. Results include bounds for solutions and existence results. Also, in some problems uniqueness of solutions was demonstrated and in other problems multiplicity of solutions was shown.

Work on epithelial transport has focused on the mathematical formulation of solute-linked water transport. An abstract analysis, applicable to a broad class of epithelial models, has been developed that assigns to any of these models an osmotic coupling coefficient. It is shown that coupling must be tight in order for a model to simulate an epithelium such as rabbit gallbladder, which can transport water both isotonically and against a substantial osmotic gradient. Previously, the compartment model of the lateral intercellular space was found to provide an adequate simulation of osmotic water transport. A "standing gradient" type model of the interspace has now been analyzed according to our general framework. We find that although the coupling may be tight for a standing gradient system, this model appears unable to realistically simulate water transport against an adverse osmotic gradient. For both the compartment and standing-gradient models, it is shown that tight coupling renders inescapable the conclusion that whole epithelial water permeability (determined experimentally) is a gross underestimate of cell membrane water permeability.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03201-20 STB

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Mathematical Theory of Renal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLBI
OTHERS:	R. Mejia	Mathematician	OD NHLBI
	B. Kellogg	Prof. IPST, University of Maryland	
	J. Garner	Prof. Louisiana Tech University, Ruston, La.	

COOPERATING UNITS (if any)

Mathematical Research Branch, NIAMDD; IPST, University of Maryland; Louisiana Tech University, Ruston, La.

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.1	0.8	0.3

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop the general theory of transport and flow processes taking place in the kidney. Aims of current work include (1) Thermodynamic and kinetic analysis of flow processes, (2) the qualitative analysis of equations describing kidney models, and (3) the development of analytical solutions of kidney models.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The primary purpose of this project is to develop the general theory of the transport and flow taking place in the kidney. This includes the thermodynamic and kinetic analysis of flow processes; the development of analytical solutions of kidney models, and the qualitative analysis of equations describing kidney models.

During the past year analytical work has been extended in two directions: (1) Mass balance in a single stage of a multistage model of the renal medulla has been analyzed. In earlier work it was shown that the mass balance equation for a variety of models of the renal medulla can be cast into the normalized form

$$r = 1/[1 - f_T(1 - f_U)(1 - f_W)],$$

where r is the ratio of total osmolality at the papilla (assumed approximately the same in all structures) to plasma osmolality, f_T is fractional solute transport out of ascending Henle's limb, f_U is the fractional urine flow, and f_W , the fractional dissipation in the vascular exchanger, is a measure of the solute that is returned to the systemic circulation by ascending vasa recta unaccompanied by its isotonic equivalent of water. This form of the mass balance equation has proved very useful in understanding the qualitative behavior of medullary models and in checking detailed calculations. With suitable interpretations of f_T, f_U, and f_W, the equation applies to multinephron models as well as single nephron models of the medulla, but only gives an overall concentration ratio. In our current work we have derived a form of the equation that is applicable to a single stage of a multistage model: Applied recursively this form permits a much finer analysis of the behavior of multistage systems.

(2) We have greatly extended our analytic solutions of medullary models. The extension results by combining a relation between volume flow, concentration, and tubal parameters in a flow tube exchanging a single neutral solute with the surrounding interstitium with the conservation equation for the renal medulla. The flow relation is

$$\frac{F_V(L)}{F_V(0)} = \left[\frac{1 + A}{r + A}\right]^{1/\sigma} ,$$

where $F_V(0)$ is volume flow entering the tube, $F_V(L)$ is flow leaving the tube, σ is the Staverman reflection coefficient, and $A = h_s/(h_V\, \sigma^2\, RT\, C_0)$, h_s being the solute permeability, h_V being the hydraulic permeability, and C_0 being the entering concentration. When this relation is combined with the normalized mass balance equation for a central core model of the medulla, we obtain

$$\frac{f}{r-1} = [\frac{1+A_1}{r+A_1}]^{1/\sigma_1} + [\frac{1+A_3}{r+A_3}]^{1/\sigma_3} \frac{F_{3v}(0)}{F_{1v}(0)}$$

This then is an equation in f, which is fractional transport rate referred to solute flow entering DHL, the reflection coefficients σ_1 and σ_3, (subscript 1 refers to DHL and 3 to CD), the composite parameters A_1 and A_3, which depend on reflection coefficients and permeabilities, and the entering volume flows $F_{3v}(0)$ and $F_{1v}(0)$. Although an analytic expression, one must in general solve it numerically, but for some special cases it can be solved analytically. Thus, for $h_{3s} = 0$ and $\sigma_1 = .5$, it reduces to a quadratic with the solution

$$r = -A + \frac{(1+A)^2}{2f} [1 \pm \sqrt{1 - \frac{4f}{1+A}}].$$

It is clear that if $4f > 1 + A$ there will be no real solution and if $4f < 1+A$, there will be two positive solutions.

We have further explored these multiple solutions numerically, utilizing a path following technique described in detail in the report on Computer Simulation of Renal Function. Their physiological significance remains to be established.

Work has continued on the qualitative analysis of equations describing kidney models. A study has been made of a number of problems, each dealing with a different set of differential equations and boundary conditions. Results include bounds for solutions and existence results. Also, in some problems uniqueness of solutions was obtained and in other problems multiplicity of solutions was shown.

A report on the earlier work of a problem for a single tube exchanging with an interstitium of known (but not constant) concentration has been recently published [1].

The analysis of the one tube problem was extended to include the case of hydrostatic pressure. Thus, a single tube exchanges water and solute with an interstitium of known (but not constant) concentration and pressure. Existence and uniqueness results are obtained for the resulting boundary value problems with certain assumptions on the transmembrane flux functions J_s and J_v. These conditions are shown to be satisfied if J_s takes the usual form of a sum of terms representing passive, active, and convective (backleak) transport. A paper [2] giving these results has been accepted for publication.

Work has continued on the analysis of a system of differential equations for a counterflow system consisting of an arbitrary number of parallel flow tubes. In the model, some tubes are allowed to have positive diffusion and other tubes have zero diffusion. The system of differential equations is of the form

$$-D_i C_i'' + (F_i C_i)' + J_{si}(x, C_1, \ldots, C_n) = 0$$

$$F_i' + J_{vi}(x, C_1, \ldots, C_n) = 0$$

where C_i, F_i denote the steady state solute concentration and volume flow, respectively, in the i^{th} tube and where J_{si}, J_{vi} denote, respectively, the total tranmembrane solute flux and volume flux out of tube i. The boundary conditions imposed are defined in terms of the geometry of the flow tubes. A paper is now in preparation that will give a general existence theorem for this system. The theorem requires certain hypotheses on the functions J_{si}, J_{vi}. These hypotheses are satisfied if there is no active transport in the system. The presence of active transport introduces certain technical difficulties that, in this general n tube problem, have not yet been overcome. Applying these results to typical flux formulas, it is shown that the system cannot concentrate with only passive and/or convective transports.

Existence and nonuniqueness results have been obtained for a boundary value problem associated with a three tube central core model of the renal medulla. Active transport is allowed out of the ascending limb. A number of solutions of the problem have been found, and a linearized stability analysis of the solutions is being undertaken. Preliminary results suggest that the dominant eigenvalue of the linearized problem, which determines the stability of the solution, will have non-zero imaginary part. A paper containing these results will be forthcoming.

Proposed Course:

We plan to continue work on the multiple solution of kidney models and will try to gain a qualitative understanding of the main features of the solution surface. Work will also be continued on the general qualitative analysis of the differential equations of the models. In the immediate future we plan to complete the work on the three-tube central core equations. Other work that will be undertaken in the near future includes: A study of the singular perturbation problems that arise when some of the permeabilities in a renal model become large; a study of numerical methods for finding the root of largest real part of a transcendental equation, especially an equation arising from the resolvant of a linear operator. The latter problem arises in our stability studies mentioned above. A satisfactory algorithm for achieving this would have application in a number of areas.

Publications:

1. Garner, J. B. and Kellogg, R. B. A One Tube Flow Problem Arising in Physiology. Bull. of Math. Biol. 42:295-304, 1980.
2. Garner, J. B. and Kellogg, R. B. The Diffusion-convection Equation With Pressure. J. Math Anal. and Applications (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER ZO HL 03202-09 STB

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Computer Simulation of Renal Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLBI
OTHERS:	R. Mejia	Mathematician	OD NHLBI
	R. Tewarson	Prof. SUNY, Stony Brook, L. I., New York	

COOPERATING UNITS (if any)

SUNY, Stony Brook, L.I., New York, and NIAMDD, Mathematical Research Branch

LAB/BRANCH

SECTION

Section on Theoretical Biophysics

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Md. 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop a computer simulation of the kidney, which describes transport of electrolyte, nonelectrolyte and water in both steady state and transient behavior. Current work is directed toward the development and theoretical analysis of efficient numerical methods of solving the differential-integral equations describing the renal counterflow system and of estimating model parameters.

Project Description:

Objectives: The purpose of this project is to develop a computer simulation of the mammalian kidney that gives a realistic description of function. This will permit the correlation of micropuncture and macroscopic clearance data with membrane transport characteristics.

Major Findings:

1. Path Following

The existence of multiple steady states for certain models of the renal medulla has been recognized by us for some time. Analysis of the equations describing central core models has shown this (J. Stephenson, R. Mejia, B. Kellogg, Multiple Solutions in Models of the Renal Medulla, FASEB abs. #560, vol. 39, Number 6, May 1, 1980). Numerical methods have been developed to (i) obtain all solutions to a model for a given set of parameters, and (ii) to follow the solution surface as a function of any one of the model's parameters.

The equations that describe solute and water movement in each tubular segment of the mammalian kidney are as follow:

$$\frac{\partial}{\partial x}\left(c_k F_v - D_k \frac{\partial c_k}{\partial x}\right) + J_k + \frac{\partial}{\partial t}(Ac_k) = As_k \quad \text{(Mass Conservation)}$$

$$\frac{\partial F_v}{\partial x} + J_v + \frac{\partial A}{\partial t} = 0 \quad \text{(Volume flow)}$$

$$\frac{\partial P}{\partial x} + RF_v = 0. \quad \text{(Pressure drop)}$$

Here x is axial distance along the tube, c_k is the concentration of the k^{th} solute, F_v is the axial volume flow, D_k is the diffusion coefficient of the k^{th} solute, J_k is the transmembrane solute flux, t is time, A is the cross sectional area of the tube, s_k is the average net rate at which material is being produced or destroyed by physical or chemical reaction, J_v is the transmembrane volume flux, P is the hydrostatic pressure, and R is the resistance to flow.

The system of such equations with the appropriate boundary conditions, which describe a model, is usually not amenable to a closed form solution. Thus, a numerical solution must be sought. The numerical method that we currently most use is one described by R. Mejia and J. L. Stephenson, An Alternating Method for Solution of a Multipoint Boundary Value Problem, SIAM, 1979 Fall Meeting, Denver, Colorado. The numerical scheme is based on that described by R. Mejia and J. L. Stephenson, Numerical Solution of Multinephron Kidney Equations, J. Computational Phys. 32 (1979), and extended to adapt the iterative method of solution to either perform a Newton step (perhaps reusing the previous Jacobian matrix) or to do a sequential secant step via a rank one update of the approximate inverse Jacobian.

However, to obtain all possible solutions for a given multipoint boundary value problem numerically is very difficult. One approach that we have found successful for a number of models (including a simple 3-tube model, a central core model of the medulla, and a full 2-nephron model) is to evaluate the homotopy map ρ on $R^n \times [0,1] \to R^n$ defined by

$$\rho(\gamma,\lambda) \equiv \lambda\phi(\gamma) - (1 - \lambda)(\gamma - \gamma_0),$$

where ϕ is the system of algebraic equations arising from discretization of the differential and conservation equations that describe the model; γ is the vector of unknown pressures, flows and solute concentrations. The initial solution, γ_0, is chosen to be readily obtained (usually known to be a solution of a problem previously solved).

We define

$$(*) \quad \frac{d\gamma}{d\lambda} + \Gamma^{-1} \frac{\partial\rho}{\partial\lambda} = 0 \qquad \text{for } \gamma(0) = \gamma_0,$$

$$\Gamma = \{\frac{\partial\rho_i}{\partial\gamma_j}\}, \qquad \frac{\partial\rho}{\partial\lambda} = \begin{pmatrix} \frac{\partial\rho_1}{\partial\lambda} \\ \vdots \\ \frac{\partial\rho_n}{\partial\lambda} \end{pmatrix}, \qquad 1 \le i, j \le n, \quad \text{and}$$

$$\phi(\gamma_0, 0) = 0.$$

If Γ has full rank, then the $\gamma(\lambda)$ obtained by solving the initial value problem defined by (*), satisfies $\rho(\gamma,\lambda) = 0$. We trace ρ very closely using an algorithm due to M. Kubiček, Dependence of Solution of Nonlinear Systems on a Parameter, Algorithm 502, ACM Transactions on Math. Software 2 (1976) and thus obtain all desired solutions, γ for which $\rho(\gamma, 1) = 0$.

Having found multiple steady states for a number of models of interest, it has remained to establish whether some solutions are modelling artifacts or have some physiological significance. Three criteria have thus far been identified for this purpose: (i) the time stability of solutions, (ii) their thermodynamic energy requirements, and (iii) the shape of the solution surface and the ability of physiologic systems to proceed from one state to another on this surface.

The models investigated thus far have had either no solution, one, two or three solutions. In the case of none or one stable steady state, the problem is solved. In the case of two or more steady states, solution of corresponding time dependent problem has often led to the identification of one solution as being unstable.

In the case of multiple stable steady states, a substantial difference in thermodynamic energy requirements (two orders of magnitude difference) in some cases leads one to believe that some steady states may be far from optimal for the kidney. However, for some models, multiple stable states have comparable energy requirements. Then the shape of the solution surface or the path from one state to another on this surface may be a means of characterizing the solutions.

To obtain a section of this surface through the λ-plane for any parameter λ, we proceed in a manner similar to the above. Define

$\rho: R^n \times R \to R^n$ by $\rho = \phi(\gamma,\lambda)$.

We then parameterize with respect to arc length, s, so that $\rho = \rho(\gamma(s),\alpha(s))$ where $\alpha = \begin{cases} \lambda & \text{or} \\ \gamma_i & \text{for some } i, \text{ where} \end{cases}$

$\gamma \equiv (\gamma_1, \gamma_2, \ldots, \gamma_n)$.

To obtain the solution surface we solve a Daviděnko type initial value problem

$$\begin{pmatrix} \frac{d\alpha}{ds} & \frac{d\gamma}{ds} \\ \frac{\partial\rho}{\partial\alpha} & \frac{\partial\rho}{\partial\gamma} \end{pmatrix} \begin{pmatrix} \frac{d\alpha}{ds} \\ \frac{d\gamma}{ds} \end{pmatrix} = \begin{pmatrix} 1 \\ 0 \end{pmatrix}$$

subject to conditions $\gamma(0) = \gamma^*$, a solution so that $\phi(\gamma^*) = 0$, and $\alpha(0) = \alpha_0$.

In some simple models this procedure has shown that transition from one steady state to another might require that parameters attain physiologically forbidden values; e.g., a negative hydraulic permeability. In addition, this makes it possible to quantitate the importance of specific parameters such as DHL and CD reflection coefficients, DHL and CD hydraulic permeabilities, and permeability requirements for passive urine concentration.

2. Vasa recta model

A six tube vasa recta model has been solved using a number of numerical schemes (R. Mejia, J. L. Stephenson and R. J. LeVeque, A Test Problem for Kidney Models, Math. Biosci. 50(1980)). It is intended to be a benchmark for investigators to validate their numerics when solving similar convection-diffusion problems. Main conclusions are the need to (1) conserve mass and water balance in the formulation and in the numerical approximations, (2) approximate the sources accurately and consistently, since small errors can lead to large changes in the solution.

3. Central Core Models

In collaboration with R. Tewarson (SUNY), research on the numerical solution of nonlinear ordinary and partial differential equations has continued.

The functional relationships in a central core model have been exploited to improve the accuracy (i.e. reduce the truncation error) of the trapezoidal rule discretization of the differential equations. This has been accomplished by first approximating the pressure equations in the tubes with local $O(h^5)$ discretizations without increasing the connectivity of the equations. Secondly, the pressure and volume flow equations in the core have been replaced by algebraic equations, thus eliminating truncation errors. Thirdly, the solute balance equations in the core have been written so that only first derivatives appear, and these are approximated using $O(h^5)$ difference equations.

This method is being tested with data derived from the full kidney multinephron model and should serve as an inexpensive, accurate, limiting case. Investigations thus far have yielded results consistent with prior trapezoidal rule implementations (locally $O(h^3)$).

Proposed Course:

Analysis of the passive concentrating mechanism using the multinephron model is expected. Various improvements of numerical techniques for solving the equation are being developed, and when successful will be incorporated into the models. Additional study of multiple steady states will be conducted.

Publications:

Mejia, R., and J. L. Stephenson. Numerical Solution of Multinephron Kidney Equations. J. Computational Phys. 32:235, 1979.

Mejia, R., J. L. Stephenson and R. J. LeVeque. A Test Problem for Kidney Models. Math. Biosci. 50:129, 1980.

Tewarson, R. P. A Numerical Method for Solving Two-Point Boundary Value Problems for Nonlinear Ordinary Differential Equations. International J. for Numerical Methods in Engineering. 15:464, 1980.

Salane, D., and R. P. Tewarson. A Unified Derivation of Symmetric quasi-Newton Update Formulas. J. Inst. Math. Appl. 25:29, 1980.

Tewarson, R. P. Solution of Nonlinear Equations Arising in the Mathematical Modeling of Large Systems. Proc. Comp. Soc. of India, Bangalore, India, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03203-05 STB

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Theory of Epithelial Transport

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	J. L. Stephenson	Chief, Section on Theoretical Biophysics	OD NHLBI
OTHERS:	A. Weinstein	Research Associate	

COOPERATING UNITS (if any)
Laboratory of Kidney and Electrolyte Metabolism, NHLBI, and NIAMDD, Mathematical Research Branch.

LAB/BRANCH

SECTION
Section on Theoretical Biophysics

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is to develop the theory of solute and water transport across epithelia. To this end, mathematical models have been developed that permit computer simulation and approximate analytic treatment of electrolyte and non-electrolyte transport in a variety of steady state and timed experiments. Current work has focused on the nature of the coupling of solute-solute and solute-solvent fluxes and on the estimation of model parameters from experimental data.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: The purpose of this project is to develop the theory of solute and water transport across epithelia. To this end comprehensive numerical models have been developed that permit computer simulation of epithelial behavior in a variety of experimental settings. It is the aim of this work to utilize such models in the design of experiments as well as in the analysis of experimental data. Further, the numerical data from these models are used in the validation of approximations employed in analytical models of transport.

Major findings: (1) An analytic framework has been developed which is suitable for characterizing and comparing the performance of various epithelial models with respect to the issues of coupled water transport. In this analysis we denote the transepithelial volume and solute fluxes by

$$J_v = J_v(C_M, C_S; C_0)$$

$$J_s = J_s(C_M, C_S; C_0),$$

where C_0 is a reference osmolality and C_M and C_S are the deviations of the mucosal and serosal bathing solutions from the reference. The tonicity of transport solution, C_R, is defined by

$$C_R = C_R(C_M, C_S; C_0) = \frac{J_s}{J_v}.$$

The ability of the epithelium to transport water against an adverse osmotic gradient is found by solving the equation

$$J_v(\hat{C}, 0; C_0) = 0$$

and $\hat{C}$ is termed the strength of transport of the model. Isotonic transport is verified by solving the equations

$$C_R(C_M^*, 0; C_0) = C_0 + C_M^*$$

and

$$C_R(0, C_S^*; C_0) = C_0 + C_S^*$$

for C_M^* and C_S^* and showing these deviations to be small fractions of C_0. (The first of these equations expresses the condition of mucosal transport equilibrium, the second, serosal transport equilibrium.) In the case that the bathing media are both at reference we denote by $(J_v)_0$ and $(J_s)_0$ the volume and solute flows and define the osmotic coupling coefficient of the model by

$$\gamma = \frac{(J_v)_0}{(J_s)_0/C_0} .$$

This formulation gains intuitive appeal when the volume and solute flows are of the simple form

$$J_v = (J_v)_0 - L_P(C_M - C_S)$$

$$J_s = (J_s)_0,$$

where L_p is the water permeability of the epithelium. In this linear model, transport isotonicity may be verified by solving for the osmotic deviation C^*, that gives transport at the reference osmolality:

$$C_R(-C^*, 0; C_0) = C_R(0, C^*; C_0) = C_0.$$

For this model,

$$C^* = \frac{((J_s)_0/C_0)(1 - \gamma)}{L_P} .$$

Thus, whereas $(J_v)_0$ is the coupled water flow that proceeds in the absence of an external gradient, an external gradient of magnitude C^* must be applied to drive the "non-coupled" fraction of water flow, $((J_s)_0/C_0)(1 - \gamma)$. Further, it is apparent that the observed L_p of the epithelium is relevant only to this non-coupled fraction of water flow. In a purely formal way, this rationalizes the observation that in some tightly coupled epithelia there are large coupled transepithelial volume flows in the face of relatively low water permeability. It is also noted that

$$\gamma = [1 + \frac{C^*}{\hat{C}}]^{-1}$$

so that for an epithelium that transports both isotonically (C^* small) and against a substantial gradient ($\hat{C}$ large) the osmotic coupling must be tight (γ close to 1).

Several existing models of epithelial water transport are of this linear form and include a compartment model of the lateral intercellular space, a standing gradient model of the interspace, and a simple epithelium between external unstirred layers. (With realistic parameter choices, only the compartment model appears capable of simulating both uphill water transport and isotonic transport.) The general analytic framework, along with the analysis of each of these epithelial models has been written up, in collaboration with Dr. Kenneth R. Spring, LKEM, NHLBI, as the chapter "Coupled Transport of Water" for the volume Membrane Transport. This material has also been presented at the Gordon Conference for Theoretical Biology (6/80).

(2) The analysis of the compartment model of the lateral intercellular space displays the composite epithelial parameters in terms of the component membrane properties. In particular, the strength of transport, $\hat{C}$, is determined by the salt transport rate relative to the channel basement membrane solute permeability, while transport isotonicity, C^*, depends on the salt transport rate relative to cell membrane water permeability, L_L. The whole epithelial L_p depends on both the cell membrane water permeability, L_L, and the channel basement membrane solute permeability and is given by

$$L_p = L_L(1 - \gamma).$$

This means that when coupling is tight, the epithelial water permeability must inevitably be a gross underestimate of the cell membrane permeability relevant to isotonic transport. Numerical studies with our comprehensive epithelial computer simulation supports the applicability of the intuition gained from this non-electrolyte, compartment analysis. This work has been submitted for publication ("Models of Coupled Salt and Water Transport Across Leaky Epithelia.")

(3) A standing-gradient-type model of the lateral interspace has been analyzed using the isotonic convection approximation of Segel. As in the compartment model, transport isotonicity depends upon the high water permeability of the cell membranes. Analagous to the compartment model, the strength of transport is enhanced by diffusion limitation within the channel, but unlike the compartment model, transport strength is weakened by high cell membrane water permeability (due to convective mixing of the channel contents). With the constraint of isotonic transport, it appears that no realistic choice of parameters can simulate the strength of transport observed in rabbit gallbladder. (Submitted for publication as "Coupled Water Transport in Standing Gradient Models of the Lateral Intercellular Space.")

Proposed Course: The isotonic convection approximation leads to a set of linear equations for epithelial transport. With these linear flux laws, the time-dependent equations for mass balance in the proximal tubule take the form of the classical wave equation. It is proposed to use the solution of the wave equation to understand data from perfused tubule experiments.

Publications:

Weinstein, A. M., and Stephenson, J. L. Electrolyte transport across a simple epithelium: Steady state and transient analysis. Biophysical J. 27:165-186, 1979.

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1979 through September 30, 1980

Investigations during the above time period as in previous years centered primarily on studies of coronary, valvular, myocardial, and congenital heart diseases, and on diseases of the lung.

CORONARY HEART DISEASE

The role of thrombus in a coronary artery in patients with fatal acute myocardial infarction remains controversial. When it was first appreciated that coronary luminal narrowing was the cause of acute myocardial infarction, it was believed that a thrombus in a coronary artery percipitated the acute myocardial infarction. In recent years from work in this branch and elsewhere it has been suggested that the thrombus in a coronary artery follows rather than percipitates an acute myocardial infarction. A group of 54 necropsy patients with transmural acute myocardial infarction and coronary thrombi were studied. Histologic sections of coronary arteries containing thrombi were examined by videoplanimetry to determine 1) the amount of luminal narrowing caused by thrombi compared to that produced by underlying atherosclerotic plaques and 2) the amount of luminal narrowing by plaques immediately proximal and distal to the thrombi. The 54 coronary arteries were narrowed by atherosclerotic plaques alone from 33% to 98% (mean 81) in cross-sectional area at the site of the thrombus; from 26-98% (mean 75) within the 2 cm segment proximal to the thrombus and from 43-98% (mean 79) within the 2 cm segment distal to the thrombus. Of the 54 coronary arteries, 52 (96%) were narrowed 76-98% in cross-sectional area by atherosclerotic plaque alone at or immediately proximal or distal to the thrombus and 26 (48%) of them were narrowed 91-98% by plaque alone. Thus, among necropsy patients with transmural acute myocardial infarction, coronary thrombi occur at sites already severely narrowed by atherosclerotic plaques. This observation suggests a relatively minor role of coronary thrombi as a terminal event in patients with fatal acute myocardial infarction.

Another study concerned the extent of coronary narrowing in patients with anterior versus posterior transmural acute myocardial infarction. It has been believed for years that patients with anterior wall infarction have more narrowing of the left anterior descending coronary artery than in either of the posterior supplying coronary arteries, and conversely that patients with posterior wall acute myocardial infarction have more narrowing of 1 or both posterior perfusing coronary artery. Each 5-mm long segment of each of the 4 major epicardial coronary arteries was studied at necropsy in 50 patients with their first acute transmural myocardial infarction. The percent of coronary arteries narrowed 76-100% in cross-sectional area by atherosclerotic plaque alone was similar in anterior and posterior wall myocardial infarction patients (74% versus 75%; average 3.0 or 4 coronary arteries per patient). A lower percentage of 5-mm coronary segments in the anterior acute myocardial infarct patients, however, were narrowed 76-100% than in posterior acute myocardial infarction (39% versus 23% [p<.001]). Thus, our necropsy patients with posterior wall myocardial infarction had more extensive and more severe narrowing than did our patients with anterior wall infarction. If the coronary arteries had not been examined in a quantitative manner, this difference in severity would not have been apparent. Also, patients with anterior wall myocardial infarction do not have more narrowing of the left anterior descending coronary artery than 1 or both posterior perfusing arteries and patients with posterior wall infarction do not have more narrowing of 1 or both posterior perfusing arteries than of the left anterior descending coronary artery.

During the last 3 years a number of quantitative coronary arterial studies have been performed in this branch. These studies have consisted of preparing a histologic section from each 5-mm segment of each of the 4 major coronary arteries. As a rule approximately 55 to 60 5-mm segments were examined from each patient. We have found that unless this quantitative approach is done that it is not possible to obtain meaningful information regarding whether one group of patients has more or less coronary narrowing than another group. One quantitative study consisted of determining the degrees of cross-sectional area luminal narrowing by atherosclerotic plaques in each 5-mm segment of each of the 4 major epicardial coronary arteries in 22 necropsy patients with systemic lupus erythematosus. Of 625 five-mm coronary segments in the lupus patients, 80 (13%) were narrowed 76-100% (controls = 0 or 431 segments); 125 (20%), 51-75% (controls = 6%); 273 (44%), 26-50% (controls = 63%) and 145 (23%), 0-25% (controls = 31%). Although the 22 patients with lupus had more coronary arterial narrowing than did the controls, 10 of the 22 lupus patients had considerably more narrowing than did the controls and the remaining 12 had similar degrees of coronary narrowing as the control patients. Comparison of the 10 lupus patients with considerable coronary narrowing to the 12 without disclosed that the former group had significantly higher mean values of total serum cholesterol, higher mean systolic/diastolic arterial pressures, and a higher frequency of pericardial disease. Thus, lupus patients treated with corticosteroids, in general, have more coronary narrowing than do control subjects but among those with lupus some have severe narrowing and others have relatively litle narrowing.

Another quantitative study consisted of examining each 5-mm segment of the 4 major epicardial coronary arteries in 6 necropsy patients with the Hurler syndrome. In 5 of the 6 patients, at least 1 of the 4 major coronary arteries was narowed 76-100%. Of the 24 major coronary arteries in the 6 patients, 17 (71%) were narrowed at some point from 76-100%. A total of 575 five-mm segments were examined from the 24 major coronary arteries and the percent narrowed to

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various degrees was as follows: 96-100 % = 14 (8 %); 76-95 % = 61 (34 %); 51-75 % = 59 (32 %); 26-50 % = 39 (21 %) and 0-25 % = 9 (5 %). Thus, narrowing of the major epicardial coronary arteries at necropsy is usually diffuse and severe in the Hurler syndrome which is the cause of the most severe degrees of coronary narrowing in childhood.

Considerable interest in recent years has centered on the usefulness of running or jogging as a preventive of coronary atherosclerosis. We studied at necropsy 5 runners who ran from 14 to 110 miles weekly (mean = 52) for 1 to 10 years (mean = 4). None had clinical evidence of cardiac disease before they became habitual runners. All died while running, and at necropsy all had severe coronary atherosclerotic narrowing. All 5, aged 14 to 53 years were white men, 4 of the 5 had hypercholesterolemia and at least 2 had systemic hypertension. Although none ever had a clinical event compatible with acute myocardial infarction, at necropsy 4 had transmural myocardial scars. Thus, among patients aged 40-55 years who are runners, it appears that coronary heart disease is the major killer. This study does not imply that running is bad for one's heart but simply that if running is to prevent development of severe coronary atherosclerosis that the running must start early in life and not after age 40.

The accuracy of determining degrees of cross-sectional narrowing of coronary arteries by visual inspection of histologic sections magnified 25 to 50 times was compared to that obtained by videoplanimetry. It was found that the degree of agreement between visual inspection of these sections under microscopy and that determined by video-planimetry, a highly accurate technique, was >95%. In addition, interobserver error was also minimal.

A group of 28 patients (24 men) with healed myocardial infarcts and true left ventricular aneurysms were examined. In contrast to other subsets of necropsy patients with fatal coronary heart disease, chronic congestive heart failure was frequent (22 patients); angina pectoris was infrequent (4 patients) and, when present, never severe; recurrence of acute myocardial infarction (2 patients), sudden death (2 patients), and clinically evident systemic emboli (1 patient) were infrequent. Additionally, most (23 patients) had large hearts (>400 gms)(mean 523 gms), 26 had dilated nonaneurysmal portions of left ventricle, and all but one had large myocardial infarcts. In 25 of the 28 patients, 2 or more of the 4 major coronary arteries were narrowed >75% in cross-sectional area by atherosclerotic plaque. Thus, the scarred, hypertrophied and aneurysmally dilated left ventricle infrequently produces chest pain or fatal arrhythmias despite severe diffuse coronary narrowing.

Although the degrees of cross-sectional area narrowing appeared to be dominant determinant of whether on e has symptons of or fatal coronary heart disease, the size of the coronary arteries in subsets of coronary heart disease patients has never been determined. Accordingly, the cross-sectional are (that portion enclosed by the

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internal elastic membrane) of histologic sections from the first 5-mm long segments of the right, left anterior descending and left circumflex coronary arteries was determined by videoplanimetry in 98 necropsy patients with fatal coronary heart disease and in 46 control subjects (without significant coronary narrowing). Significant ($p < 0.002$) differences were observed in mean cross-sectional area of each of the 3 major coronary arteries in 5 subgroups of coronary patients and among and between the control subjects and these differences resulted primarily from differences in heart weight and to a slight extent differences in age. Therefore, the coronary size and myocardial mass cancel out one another and one is left with cross-sectional area narrowing as the major determinant of symptons or fatality from coronary heart diesease.

It is been believed for many years that the epicardial coronary arteries have accelerated degrees of atherosclerosis in patients with diabetes mellitus compared to those without this metabolic distrubance. We studied 229 necropsy patients with diabetes mellitus with onset of diabetes after 30 years of age: 65 of them had no symptomatic coronary heart disease and 164 had clinical evidence of coronary heart disease. These 229 necropsy patients were compared to 183 age, sex matched non-diabetic patients who died from a fatal coronary event. The average number of 3 major coronary arteries per patient narrowed 76-100% in cross-sectional area by atherosclerotic plaque was similar in the 229 diabetic patients and in the 183 non-diabetic patients. This similarity in the amount of coronary arterial narrowing was present irrespective of the age at onset (after 30 years) or duration of the diabetes mellitus. The diabetic patients with symptomatic coronary heart disease had more severe narrowing of the 3 major coronary arteries then did the diabetic patients without symptomatic coronary heart disease ($p < .01$).

During the last 10 years a number of angiographic studies have emphasized the poor prognosis in patients with severe narrowing of the left main coronary artery but no studies have appeared examining the accuracy of angiographic interpretation of luminal narrowing of this artery. To evaluate the accuracy of coronary angiography in identifying severe narrowing of the left main coronary artery, the degree of narrowing observed by angiography was compared to that of observed at necropsy in 28 patients with symptomatic coronary heart disese in whom angiography had been performed within 40 days of death. The angiograms were evaluated independently by 3 experienced angiographers. In 20 (71%) of the 28 patients, the degree of narrowing of the left main coronary artery was either underestimated (13 patients) or overestimated (10 patients) by 2 or 3 of 3 angiographers: of 84 angiographic judgments made by the 3 angiographers in the 28 patients, 54 (64%) were underestimates (33 judgments [39%]) or overestimates (21 judgments [25%]) of the degree of left main coronary narrowing. Thus, angiographic determinations of degree of narrowing of this artery during life is subject to considerable error.

In our study of chronic true left ventricular aneurysm, we observed a very low frequency of systemic emboli despite a very high frequency of intraaneurysmal thrombus. From previous studies in this branch we have also observed that thrombi are frequent in the left ventricle and other cardiac chambers in patients with idiopathic dilated cardiomyopathy and that systemic emboli in the latter group of patients are relatively frequent. Therefore, we attempted to determine why systemic emboli were infrequent in the patients with true left ventricular aneurysm and frequent in those with idiopathic dilated cardiomyopathy. It was reasoned that emboli in the patients with left ventricular aneurysm was infrequent because the thrombus was present only within the aneurysm and therefore only one of its 4 surfaces was exposed to the intracavitary left ventricular blood. In contrast, in patients with idiopathic dilated cardiomyopathy, the left ventriclar wall beneath the thrombus was not scarred or necrotic or bugled and, therefore, at least 3 of its surfaces were exposed to the intracavitary blood.

VALVULAR HEART DISEASE

Although the Björk-Shiley prosthesis has many favorable features, it is a delicate prosthesis and therefore is subject to complications which might not complicate valve replacement with other prostheses, like the caged ball in which the margin of error is less. We studied 3 patients who developed severe aortic regurgitation after aortic valve replacement with a tilting-disc prosthesis. The regurgitation, fatal in 2, resulted from long sutures or their knots or both overhanging the metalic ring of the prosthesis, preventing complete seating of the occluder. In 1 patient simply cutting the overhanging suture eliminated the aortic regurgitation. The problem of suture overhang appears more lightly to occur when aortic valve replacement is carried in patients with relatively small aortic roots.

Evaluation of porcine bioprostheses has been a continuing interest of this branch. Of 14 porcine bioprostheses found to contain calcific deposits at the time of removal, either at reoperation (13 patients) or necropsy (1 patient), 11 had been in the mitral position, 1 in the aortic, 1 in the tricuspid, and 1 in a pulmonary arterial conduit. The bioprostheses had been in place from 3 to 94 months (mean 39). From analysis of these 14 bioprostheses and review of reports concerning 37 other calcified porcine bioprostheses, the following conclusions were made: calcific deposits may occur in all aged patients but are more likely to become severe and clinically significant in children and in young adults than in older patients; the calcific deposits in bioprostheses can cause the substitute valve to become stenotic or incompetent; metabolic disorders in the patients with bioprostheses were not found; the 2 main sites of calcific deposition in these porcine valve bioprostheses were the connective tissue in the cusps and small thrombi on the cuspal surfaces.

MYOCARDIAL HEART DISEASE

It is well recognized that myocardial fiber disorganization is a common histologic feature of patients with hypertrophic cardiomyopathy. We compared the extent of the myocardial-cell disorganization in excised operative septectomy specimens to that observed at necropsy in the same 18 patients with hypertrophic cardiomyopathy who died after operation. Disorganization was present in all 18 patients at necropsy and was extensive (>5%) in 16 of them. In contrast, there was no disorganziation in the operative specimens of 3 patients and only a trivial amount (<1%) in 5 others. Disorganization extensive enough (>5%) to suggest hypertrophic cardiomyopathy was present in the operative specimens of only 6 of the 18 patients. The mean area of disorganization at necropsy was 20 ± 3% compared to 7 ± 2% in the operative specimens ($p<0.001$). Thus, histologic examination of operatively excised septectomy specimens is of limited value in confirming the diagnosis of hyperthrophic cardiomyopathy.

There are many causes of sudden cardiac death. The most common in our experience of course being coronary heart disease and secondly hypertrophic cardiomyopathy. Among young individuals we found several in whom sudden death occurred unexpectedly and the diagnosis at necropsy was cardiac sarcoidosis. This condition should be added to the list of causes of sudden cardiac death, particularly in young patients.

The Ultrastructure Section of the Pathology Branch has particularly been interested in recent years in adriamycin toxicity and the effect of certain compounds on that toxicity. In dogs, rabbits, and pigs the effects of vitamin E and selenium on adriamycin toxicity was investigated and cardioprotection by vitamin E and or selenium was found to be of limited value or nonexistent.

CONGENITAL HEART DISEASE

Most patients with congenital heart disease have defects in atrial or ventricular septum or obstructions at or below or above cardiac valves or anomalies of arterial or venous connections or combinations of the 3. During the past year, we studied 2 patients at necropsy in whom a portion of both right and left ventricular free wall was hypoplastic from birth. This parchment-like thinning of portions of the right and left ventricular free wall (called by some Uhl's anamoly) was found in a 61-year-old man who died suddenly but had never previously had evidence of cardiac dysfunction and in a 55-year-old woman who had progressive eventually intractable fatal congestive heart failure of 29 months duration. Thus, patients with parchment-like hearts may have involvment of the left ventricular free wall as well as the right. While some have no evidence of cardiac dysfunction others develop severe progressive congestive failure.

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We also studied 2 patients with pulmonic valve stenosis, atrial septal defect and left-to-right interatrial shunting with intact ventricular septa. It is well known that patients with pulmonic valve stenosis often have right-to-left shunting at the atrial level but it has not been explained why patients with pulmonic valve stenosis may at times have pure left-to-right shunting. The 2 patients we studied were 69 and 55 years of age, respectively. Both had moderate degrees of pulmonic valve obstruction (pressure gradient about 55 mm Hg) and large secundum type atrial septal defects. In comparison to previous patients reported from this branch with right-to-left shunting at the atrial level associated with pulmonic valve stenosis, the patients with pure left-to-right shunts at the atrial level had large secundum atrial septal defects and only moderate pulmonic valve stenosis whereas those with right-to-left shunts had patent foramen ovale only and severe pulmonic valve obstruction.

It well known that patients with the Marfan syndrome usually die from a distinctive cardiovascular malformation affecting mainly the ascending aorta and that the average age of death is 34 years. We studied a 69-year-old man who had none of the skeletal features of the Marfan syndrome but yet had all of the distinctive cardiovascular features. Why some patients with the Marfan type of cardiovascular disease live into the 6th, 7th and 8th decades and why other patients with similar cardiovascular Marfan type malformations but without the Marfan syndrome die at a relatively young age has not been determined.

PERICARDIAL HEART DISEASE

The structure of normal human parietal pericardium has not been examined in detail. Such was the case carried out in 7 patients. It was observed that the normal human parietal pericardium is composed of 3 layers: the serosa, the fibrosa and the epipericardial connective tissue layer. Scanning electron microscopic examination of parietal pericardium disclosed the pericardial mesothelial cells to have single cilia with microvilli. The latter appear to bear friction and increase the surface area for fluid transport. Junctional complex between adjacent mesothelial cells were found to consist of desmosomes, which reinforce intercellular adhesion, and zonulae occludentes, which form permeability barriers. The collagen in the fibrosis has a wavy or "crimped" configuration which permits stretching of the pericardial connective tissue.

MISCELLANEOUS HEART DISEASE

Experimentally it has been demonstrated that radiation in high doses can damage the heart in non-human experimental animals but surprisingly relatively little morphologic information is available on the effect of radiation on human heart. We studied 16 necropsy patients aged 15 to 33 years who received >3500 rad to the heart 5-144

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months before death. All had some radiation-induced damage to the heart: 15 had thickened pericardia; 8 had increased interstitial myocardial fibrosis, particularly in the right ventricle; 12 had fibrous thickening of the mural endocardium and 13, of the valvular endocardium. Except for valvular thickening, the changes were more frequent in the right than in the left side of the heart, presumably because of higher radiation doses to the anterior cardiac surface. Six of the 16 study patients and 1 of 10 control subjects had 1 or more major epicardial arteries narrowed 76-100% in cross-sectional area by atherosclerotic plaque. Thus, radiation to the heart may produce a wide spectrum of functional and anatomical changes, but particularly damage to the pericardia and the underlying epicardial coronary arteries.

The cardiac lesions produced in dogs by single transthoracic damped sinusoidal waveform shocks were examined and these were found to consist of focal area of myocyte necrosis with contraction bands and calcific deposits. Inflammatory cells also were present. The lesions underwent gradual resolution over a period of 8 weeks.

Fatal Petriellidium Boydii infection with right-sided endocarditis was described in a 62 year old woman who had mixed connective tissue disease treated by cordicosteroids. Endocarditis due to P. Boydii has not been described previously.

LUNG

A study was done on subplasmalemmal linear densities and mononuclear cells found in the lung. These densities consisted of a thin layer of electron-dense material immediately subjacent to the inner aspect of the plasma membrane and they were found in cells of the mononuclear phagocyte system in lung biopsies from patients with fibrotic disorders. Two types of subplasmalemmal linear densities were found.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03166-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Pulmonic-Valve Stenosis, Atrial Septal Defect and Left-To-Right Interatrial Shunting With Intact Ventricular Septum

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Ernest N. Arnett, Medicine Department, Franklin Square Hospital, Baltimore, Maryland

OTHER: Seena C. Aisner, Pathology Department, Franklin Square Hospital, Baltimore, Maryland
Kenneth B. Lewis, Medicine Department, Franklin Square Hospital, Baltimore, Maryland
Paul Tecklenberg, Medicine Department, Franklin Square Hospital, Baltimore, Maryland
Robert K. Brawley, Department of Surgery, The Johns Hopkins Hospital, Baltimore, Maryland
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Medicine and Pathology Departments, Franklin Square Hospital, Baltimore,MD
Department of Surgery, The Johns Hopkins Hospital, Baltimore, MD

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Described in this report are clinical and morpholoic features in two patients with pulmonic-valve stenosis with secundum type atrial septal defect and large left-to-right shunts at the atrial level. One of the patients was a 69-year-old woman who had been asymptomatic until sudden cardiac arrest and the second a 55-year-old woman who became asymptomatic with evidence of congestive heart failure at age 48. Although it is well known that patients with pulmonic stenosis and atrial septal defect commonly have right-to-left shunts at the atrial level. It is less well appreciated that some of these patients have pure left-to-right shunts as described in the two patients in this report. The explanation for the differening direction of the shunting at the atrial level is the size of the defect in the atrial septum and the degree of obstruction to right ventricular outflow.

PHS-6040
(Rev. 10-76)

Project Description: Described in this report are clinical and morpholoic features in two patients with pulmonic-valve stenosis with secundum type atrial septal defect and large left-to-right shunts at the atrial level. One of the patients was a 69-year-old woman who had been asymptomatic until sudden cardiac arrest and the second a 55-year-old woman who became asymptomatic with evidence of congestive heart failure at age 48. Although it is well known that patients with pulmonic stenosis and atrial septal defect commonly have right-to-left shunts at the atrial level. It is less well appreciated that some of these patients have pure left-to-right shunts as described in the two patients in this report. The explanation for the differening direction of the shunting at the atrial level is the size of the defect in the atrial septum and the degree of obstruction to right ventricular outflow. Comparison to 19 previously reported patients from this laboratory with pulmonic valve stenosis with atrial septal defect and right-to-left shunting at the atrial level disclosed that those with pure right-to-left shunting had true atrial septal defects of the secundum type whereas the patients with right-to-left shunt had only patent foramen ovales and the patients with only left-to-right shunts had only moderate degrees of pulmonic-valve obstruction (mean gradient 60 mm Hg) whereas those with right-to-left shunts had severe pulmonic stenosis (mean gradients 120 mm Hg).

Publication: Arnett, E.N., Aisner, S.C., Lewis, K.B.,Tecklenberg, P., Brawley, R.K., Roberts, W.C.: Pulmonic-Valve Stenosis, Atrial Septal Defect and Left-to-Right Interatrial Shunting With Intact Ventricular Septum. A Distinct Hemodynamic-Morphologic Syndrome. CHEST (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03167-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Cardiac Sarcoidosis. A Major Cause of Sudden Death in Young Individuals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Renu Virmani, Staff Fellow, PB, NHLBI

OTHER: J. Conrad Bures, Department of Pathology, Montefiore Hospital, University of Pittsburgh Health Center, Pittsburgh, Pennsylvania
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any) Deaprtment of Pathology, Montefiore Hospital, University of Pittsburgh Health Center, Pittsburgh, Pennsylvania

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS x☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This report describes a 35-year-old man who, during his last few months of life, was noted to have periodic premature ventricular contractions and progressive enlargement on chest roentgenogram. He died suddenly and unexpectedly at work. At necropsy the heart was infiltrated by numerous sarcoid granulomas. The purpose of the report was to point out that sudden death in young individuals may be the result of cardiac sarcoidosis and a number of patients previously recorded with this situation were reviewed.

PHS-6040
(Rev. 10-76)

Project Description: This report describes a 35-year-old man who, during his last few months of life, was noted to have periodic premature ventricular contractions and progressive enlargement on chest roentgenogram. He died suddenly and unexpectedly at work. At necropsy the heart was infiltrated by numerous sarcoid granulomas. The purpose of the report was to point out that sudden death in young individuals may be the result of cardiac sarcoidosis and a number of patients previously recorded with this situation were reviewed.

Publications: Virmani, R., Bures, J.C., and Roberts, W.C.: Cardiac sarcoidosis. A major cause of sudden death in young individuals. Chest. 77:423-428, March 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03168-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Coronary Arterial Disease in the Hurler Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frank C. Brosius, III, Staff Fellow, PB, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

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☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The amount of cross-sectional area luminal narrowing in each five-mm segment of each of the four major (right, left main, left anterior descending and left circumflex) epicardial coronary arteries is described at necropsy in six children (aged 3-16 years) with the Hurler syndrome. In five of the six patients at least one of the four major coronary arteries was narrowed 76-100 percent, and in four of these five patients all four major arteries were narrowed to this extent. Of the 24 major coronary arteries in the six patients, 17 (71 percent) were narrowed at some point from 76-100 percent. A total of 570 five-mm segments were examined from the 24 major coronary arteries and the percent narrowed to various degrees was as follows: 96-100 percent = 14 (8 percent); 76-95 percent = 61 (34 percent); 51-75 percent = 59 (32 percent); 26-50 percent = 39 (21 percent) and 0-25 percent = 9 (5 percent).

Project Description: The amount of cross-sectional area luminal narrowing in each five-mm segment of each of the four major (right, left main, left anterior descending and left circumflex) epicardial coronary arteries is described at necropsy in six children (aged 3-16 years) with the Hurler syndrome. In five of the six patients at least one of the four major coronary arteries was narrowed 76-100 percent, and in four of these five patients all four major arteries were narrowed to this extent. Of the 24 major coronary arteries in the six patients, 17 (71 percent) were narrowed at some point from 76-100 percent. A total of 570 five-mm segments were examined from the 24 major coronary arteries and the percent narrowed to various degrees was as follows: 96-100 percent = 14 (8 percent); 76-95 percent = 61 (34 percent); 51-75 percent = 59 (32 percent); 26-50 percent = 39 (21 percent) and 0-25 percent = 9 (5 percent). By applying a score of one to four to each five-mm segment according to its category of narrowing (1 = 0-25 percent; 2 = 26-50 percent; 3 = 51-75 percent and 4 = 76-100 percent), the 182 segments had a total score of 570 and a mean score of 3.2, indicating that each segment was narrowed on the average about 67 percent in cross-sectional area. Thus, narrowing of the major epicardial coronary arteries at necropsy is usually diffuse and severe in the Hurler syndrome which is the cause of the most severe degrees of coronary narrowing in childhood.

Publication: Brosius, F.C. III, Roberts, W.C.: Coronary Arterial Disease in the Hurler Syndrome. A Qualitative and Quantitative Analysis of the Extent of Coronary Narrowing at Necropsy in 6 Children. Am J Cardiol (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03169-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Marfan Cardiovascular Disease without the Marfan Syndrome

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, PB, NHLBI

OTHER: Robert L. Reis, Surgery Branch, NHLBI
Charles L. McIntosh, Surgery Branch, NHLBI
Stephen E. Epstein, Cardiology Branch, NHLBI
William C. Roberts, Chief, PB, NHLBI

COOPERATING UNITS (if any)

Surgery Branch, NHLBI
Cardiology Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Described in this report is a 69-year-old man who had all of the cardiovascular features of the Marfan Syndrome yet he had none of the skeletal or familial historical features of the Marfan Syndrome. Management of the cardiovascular consequences associated with this syndrome are discussed in this report.

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PHS-6040
(Rev. 10-76)

Project Description: Described in this report is a 69-year-old man who had all of the cardiovascular features of the Marfan Syndrome yet he had none of the skeletal or familial historical features of the Marfan Syndrome. Management of the cardiovascular consequences associated with this syndrome are discussed in this report.

Publications: Waller, B.F., Reis, R.L., McIntosh, C.L., Epstein, S.E., and Roberts, W.C.: The Marfan cardiovascular disease without the Marfan Syndrome. Chest. 77:533-540, April 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03170-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Congenital Heart Disease with Trisomy 13

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry S. Cabin, Clinical Associate, NHLBI

OTHER: Lucille A. Lester, Clinical Associate, NIAMDD
William C. Roberts, Chief, PB, NHLBI

COOPERATING UNITS (if any)

NIAMDD

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Described in this report is a 4-month-old girl who had all of the clinical and morphologic features of Trisomy 13. These consequences are described and summarized and the means of differentiating ventricular septal defect from atrial septal defect by echocardiogram are described.

PHS-6040
(Rev. 10-76)

Project Description: Described in this report is a 4-month-old girl who had all of the clinical and morphologic features of Trisomy 13. These consequences are described and summarized and the means of differentiating ventricular septal defect from atrial septal defect by echocardiogram are described.

Publications: Cabin, H.S., Lester, L.A., and Roberts, W.C.: Congenital heart disease with Trisomy 13. American Heart Journal. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03171-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Morphologic Features of Certain Myocardial Complications of Acute Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This article reviews the myocardial complications of acute myocardial infarction namely cardiogenic shock, papillary muscle dysfunction, rupture of the ventricular septum, rupture of the left ventricular free wall, true left ventricular aneurysm and coronary dilated cardiomyopathy.

Project No. Z01 HL 03171-01 PA

Project Description: This article reviews the myocardial complications of acute myocardial infarction namely cardiogenic shock, papillary muscle dysfunction, rupture of the ventricular septum, rupture of the left ventricular free wall, true left ventricular aneurysm and coronary dilated cardiomyopathy.

Publications: Roberts, W.C.: Morphologic features of certain myocardial complications of acute myocardial infarction. In Surgery for the Complications of Myocardial Infarction. Edited by John M. Moran, M.D. and Lawrence L. Michaelis, M.D. Grune & Stratton, Inc., 1980 (June), pp. 1-24.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03172-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Iatrogenic Pulmonary Granulomatosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, PB, NHLBI

OTHER: W. J. Brownlee, Pathologist, D.C. Medical Examiner's Office
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Described in this report is an 18-year-old girl who took heroin injections intravenously for the last three years of her life and during the last twelve months of life developed periodic wheezing and exertional dyspnea which progressed. She died suddenly at home and necropsy disclosed numerous talc containing granulomas in the lungs.

835

PHS-6040
(Rev. 10-76)

Project Description: Described in this report is an 18-year-old girl who took heroin injections intravenously for the last three years of her life and during the last twelve months of life developed periodic wheezing and exertional dyspnea which progressed. She died suddenly at home and necropsy disclosed numerous talc containing granulomas in the lungs. Because it holds the components of a pill together, talc is present in virtually all tablets and consequently if tablets of nearly any variety are dissolved in a fluid and injected into systemic veins rather than swallowed, pulmonary talc granulomas are consequences. The patient described crushed tablets and dissolved them and then injected them into her veins and developed these multiple talc granulomas. The pulmonary consequences are discussed in this report.

Publications: Waller, B.F., Brownlee, W.J., and Roberts, W.C.: Iatrogenic pulmonary granulomatosis. Chest. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03173-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Disappearance of Symptomatic Coronary Heart Disease and Death from a Noncardiac Condition

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Renu Virmani, Staff Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A 55-year-old man who had the onset of angina pectoris at age 50 is described. The angina persisted for three and a half years and then disappeared spontaneously and the patient died from a non-cardiac condition, namely cancer. The purpose of this report was to describe the amount of narrowing in the coronary arteries in a patient who had symptomatic coronary heart disease but then died of a non-cardiac condition. It was found that the amount of narrowing was similar to that previously observed in patients with fatal symptomatic coronary heart disease.

Project Description: A 55-year-old man who had the onset of angina pectoris at age 50 is described. The angina persisted for three and a half years and then disappeared spontaneously and the patient died from a non-cardiac condition, namely cancer. The purpose of this report was to describe the amount of narrowing in the coronary arteries in a patient who had symptomatic coronary heart disease but then died of a non-cardiac condition. It was found that the amount of narrowing was similar to that previously observed in patients with fatal symptomatic coronary heart disease.

Publications: Virmani, R., Roberts, W.C.: Disappearance of symptomatic coronary heart disease and death from a noncardiac condition. Chest. 77:91-93, January 1980.

MITHSONIAN SCIENCE INFORMATION EXCHANGE ROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03174-01 PA

ERIOD COVERED

October 1, 1979 to September 30, 1980

ITLE OF PROJECT (80 characters or less)

Death in the Disco

AMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER ROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frank C. Brosius, III, Staff Fellow, PB, NHLBI

OTHER: Brian D. Blackbourne, Pathologist, D.C. Medical Examiner's Office, Washington, D.C.
William C. Roberts, Chief, PB, NHLBI

COOPERATING UNITS (if any)

D.C. Medical Examiner's Office
Washington, D.C.

AB/BRANCH

Pathology Branch

ECTION

NSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

OTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

HECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Described in this report is a 31-year-old white man who died suddenly in a disco. Although he had been asymptomatic prior to that time, in retrospect he had evidence of type II hyperlipoproteinemia. This report points out that the most common cause of death in individuals in their fourth decade of life is coronary heart disease.

PHS-6040

Project Description: Described in this report is a 31-year-old white man who died suddenly in a disco. Although he had been asymptomatic prior to that time, in retrospect he had evidence of type II hyperlipoproteinemia. This report points out that the most common cause of death in individuals in their fourth decade of life is coronary heart disease.

Publications: Brosius, III, F.C., Blackbourne, B.D., and Roberts, W.C.: Death in the disco. Chest. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03175-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Left Ventricular Aneurysm, Intraaneurysmal Thrombus and Systemic Embolus in Coronary Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry Scott Cabin, Clinical Associate, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This paper describes the problem in many studies concerning left ventricular aneurysm and what is the definition of aneurysm. A number of reports were described in which the definition of aneurysm was never mentioned or was imprecise. This study also asks the question as to why systemic embolus was uncommon in patients with left ventricular aneurysm secondary to coronary heart disease because intraaneurysmal thrombus in these individuals was so common. It was speculated that the reason emboli in these patients with aneurysm was so infrequent was that the thrombus was present in the aneurysm alone and therefore only one surface of the thrombus was exposed to the intra left ventricular cavity blood.

841

Project Description: This paper describes the problem in many studies concerning left ventricular aneurysm and what is the definition of aneurysm. A number of reports were described in which the definition of aneurysm was never mentioned or was imprecise. This study also asks the question as to why systemic embolus was uncommon in patients with left ventricular aneurysm secondary to coronary heart disease because intraaneurysmal thrombus in these individuals was so common. It was speculated that the reason emboli in these patients with aneurysm was so infrequent was that the thrombus was present in the aneurysm alone and therefore only one surface of the thrombus was exposed to the intra left ventricular cavity blood. In other words, the thrombus did not bulge into the left ventricular cavity as it did in patients, for example, with dilated cardiomyopathy and in the latter group systemic emboli are very frequent.

Publications: Cabin, H.S., and Roberts, W.C.: Left ventricular aneurysm, intraaneurysmal thrombus and systemic embolus in coronary heart disease. Chest. 77:586-590, May 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER **Z01 HL 03176-01 PA**

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Melanoma to the Cor

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI

OTHER: John S. Gottdiener, Senior Investigator, Cardiology Branch, NHLBI
Renu Virmani, Staff Fellow, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland

TOTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This report describes a patient with melanoma to the heart and the patient developed pericardial fusion. The purpose of the report was to discuss proper management of patients with cardiac metastases.

843

PHS-6040
(Rev. 10-76)

Project Description: This report describes a patient with melanoma to the heart and the patient developed pericardial fusion. The purpose of the report was to discuss proper management of patients with cardiac metastases.

Publications: Waller, B.F., Gottdiener, J.S., Virmani, R., and Roberts, W.C.: Melanoma to the Cor. Chest. 77:671-676, May 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03177-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Accuracy of angiographic determination of left main coronary arterial narrowing

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. M. Isner, Staff Associate, PB, NHLBI

OTHER: J. Kishel, Student, George Washington University School of Medicine, Washington, D.C.
K. M. Kent, Chief,
J. A. Ronan, Cardiologist, Seventh Day Adventist Hospital, Takoma Park, Maryland
A. M. Ross, Chief, Division of Cardiology, George Washington University Hospital, Washington, D.C.
W. C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

1) Cardiology Branch, NHLBI
2) Department of Medicine, G.W. University, Washington, D.C.
3) Department of Cardiology, Seventh Day Adventist Hospital, Takoma Park, MD

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

To evaluate the accuracy of coronary angiography in identifying severe narrowing of the left main coronary artery (LMCA), the degree of narrowing observed by angiography was compared to that observed at necropsy in 28 patients with symptomatic coronary heart disease in whom angiography had been performed within 40 days of death. The angiograms were evaluated independently by 3 experienced angiographers. In 20 (71%) of the 28 patients, the degree of narrowing of the LMCA was either underestimated (13 patients) or overestimated (10 patients) by 2 or 3 of 3 angiographers; of 84 angiographic judgments made by the 3 angiographers in the 28 patients, 54 (64%) were underestimates (33 judgments [39%]) or overestimates (21 judgments [25%]) of the degree of LMCA narrowing.

Project Description: To evaluate the accuracy of coronary angiography in identifying severe narrowing of the left main coronary artery (LMCA), the degree of narrowing observed by angiography was compared to that observed at necropsy in 28 patients with symptomatic coronary heart disease in whom angiography had been performed within 40 days of death. The angiograms were evaluated independently by 3 experienced angiographers. In 20 (71%) of the 28 patients, the degree of narrowing of the LMCA was either underestimated (13 patients) or overestimated (10 patients) by 2 or 3 of 3 angiographers; of 84 angiographic judgments made by the 3 angiographers in the 28 patients, 54 (64%) were underestimates (33 judgments [39%]) or overestimates (21 judgments [25%]) of the degree of LMCA narrowing. Of 12 LMCAs narrowed 76-100% in cross-sectional area (XSA) at necropsy, 6 were underestimated at preoperative angiography by 2 or 3 of 3 angiographers; of 12 LMCAs narrowed 51-75% in XSA at necropsy, all 12 were either under- or over-estimated angiographically by 2 or 3 of 3 angiographers; of 4 LMCAs narrowed 26-50% in XSA at necropsy, 2 were overestimated by 2 of 3 angiographers. Thus, angiographic determination of degrees of narrowing of the LMCA during life is subject to considerable error. The angiographic errors appear to have resulted primarily from 1) the presence of atherosclerotic plaque in the LMCA in all 28 patients and 2) an insufficient number of angiographic projections in all 28 patients.

Publications: Isner, J.M., Kishel, J., Kent, K.M., Ronan, J.A., Ross, A.M., Roberts, W.C.: Accuracy of angiographic determination of left main coronary arterial narrowing: Angiographic-histologic correlative analysis in 28 patients. Circulation. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03178-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

True Left Ventricular Aneurysm and Healed Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry S. Cabin, Clinical Associate, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy observations are described in 28 patients (24 men) aged 31-85 years (mean 62) with healed myocardial infarcts and true left ventricular aneurysms. In contrast to other subsets of necropsy patients with fatal coronary heart disease, chronic congestive cardiac failure was frequent (22 patients); angina pectoris was infrequent (4 patients) and, when present, never severe; recurrence of acute myocardial infarction (2 patients), sudden death (2 patients), and clinically evident systemic emboli (1 patient) were infrequent; survival for more than 5 years after healing of the acute myocardial infarct was infrequent (in 3 of 21 patients with clinically diagnosed acute myocardial infarcts), and survival for longer than 1 year after aneurysmectomy was lacking (0 of 7 patients). Additionally, most (23 patients) had large hearts (greater than 400gms) (mean 523), 26 had dilated nonaneurysmal portions of left ventricle, and all but one had large (greater than 30% of the left ventricular wall) myocardial infarcts. In 25 of the 28 patients, 2 or more of the 4 major epicardial coronary arteries were greater than 75% narrowed in cross-sectional area by atherosclerotic plaques.

PHS-6040
(Rev. 10-76)

Project Description: Clinical and necropsy observations are described in 28 patients (24 men) aged 31-85 years (mean 62) with healed myocardial infarcts and true left ventricular aneurysms. In contrast to other subsets of necropsy patients with fatal coronary heart disease, chronic congestive cardiac failure was frequent (22 patients); angina pectoris was infrequent (4 patients) and, when present, never severe; recurrence of acute myocardial infarction (2 patients), sudden death (2 patients), and clinically evident systemic emboli (1 patient) were infrequent; survival for more than 5 years after healing of the acute myocardial infarct was infrequent (in 3 of 21 patients with clinically diagnosed acute myocardial infarcts), and survival for longer than 1 year after aneurysmectomy was lacking (0 of 7 patients). Additionally, most (23 patients) had large hearts (greater than 400gms) (mean 523), 26 had dilated nonaneurysmal portions of left ventricle, and all but one had large (greater than 30% of the left ventricular wall) myocardial infarcts. In 25 of the 28 patients, 2 or more of the 4 major epicardial coronary arteries were greater than 75% narrowed in cross-sectional area by atherosclerotic plaques. Of 992 five-mm long segments of major coronary artery examined in 22 patients (45 segments per patient), 323 (33%) were narrowed greater than 75% in cross-sectional area by atherosclerotic plaques; 419 (42%), 51-75%; 210 (21%), 26-50%, and 40 (4%), 0-25%. Thus, the scarred, hypertrophied and aneurysmally dilated left ventricle infrequently produces chest pain or fatal arrhythmia despite diffuse, severe coronary narrowing.

Publications: Cabin, H.S., Roberts, W.C.: True left ventricular aneurysm and healed myocardial infarction: Clinical and necropsy observations including quantification of degrees of coronary arterial narrowing. Americal Journal of Cardiology. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03179-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparison of amount of myocardial-cell disorganization in operatively-excised septectomy specimens with amount observed at necropsy in 18 patients with hypertrophic cardiomyopathy

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. M. Isner, Staff Associate, PB, NHLBI

OTHER: B. J. Maron, Senior Investigator, Cardiology Branch, NHLBI
W. C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The extent of myocardial-cell disorganization in excised septectomy specimens was compared morphometrically to that observed at necropsy in the same 18 patients with hypertrophic cardiomyopathy who died after operation. Disorganization was present in all 18 patients at necropsy and was extensive ($\geq$ 5%) in 16 of the 18. In contrast, there was no disorganization in the operative specimens of 3 patients and only a trivial amount (< 1%) in 5 others. Disorganization extensive enough ($\geq$ 5%) to suggest hypertrophic cardiomyopathy was present in the operative specimens of only 6 of the 18 patients. The mean area of disorganization at necropsy was 20 ± 3% compared to 7 ± 2% in the operative specimens ($p < 0.001$).

PHS-6040
(Rev. 10-76)

Project Description: The extent of cardiac-muscle-cell disorganization in excised septectomy specimens was compared morphometrically to that observed at necropsy in the same 18 patients who died after operation. Disorganization was present in all 18 patients at necropsy and was extensive ($\geq 5\%$) in 16 of the 18. In contrast, there was no disorganization in the operative specimens of 3 patients and only a trivial amount ($< 1\%$) in 5 others. Disorganization extensive enough ($\geq 5\%$) to suggest hypertrophic cardiomyopathy was present in the operative specimens of only 6 of the 18 patients. The mean area of disorganization at necropsy was 20 ± 3% compared to 7 ± 2% in the operative specimens ($p < 0.001$). Thus, histologic examination of operatively excised septectomy specimens is of limited value in confirming the diagnosis of hypertrophic cardiomyopathy.

Publications: Isner, J.M., Maron, B.J., Roberts, W.C.: Comparison of amount of cardiac-muscle-cell disorganization in operatively-excised septectomy specimens to amount observed at necropsy in the same 18 patients with hypertrophic cardiomyopathy. American Journal of Cardiology. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03180-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Post-operative Aortic Regurgitation from Incomplete Seating of Tilting-disc Occluders Due to Overhanging Knots or Long Sutures

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI
Other: Michael Jones, Senior Investigator, Surgery Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Certain clinical and morphologic features are described in 3 patients who developed severe aortic regurgitation after aortic valve replacement. The regurgitation, fatal in 2 of the 3 patients, resulted from long sutures or their knots or both overhanging the metallic ring of the prosthesis preventing complete seating of the occluder. In one patient simply cutting the overhanging suture eliminated the aortic regurgitation. The problem of suture overhang appears more likely to occur when aortic valve replacement is carried out in patients with relatively small aortic roots.

PHS-6040
(Rev. 10-76)

Project Description: Certain clinical and morphologic features are described in 3 patients who developed severe aortic regurgitation after aortic valve replacement. The regurgitation, fatal in 2 of the 3 patients, resulted from long sutures or their knots or both overhanging the metallic ring of the prosthesis preventing complete seating of the occluder. In one patient simply cutting the overhanging suture eliminated the aortic regurgitation. The problem of suture overhang appears more likely to occur when aortic valve replacement is carried out in patients with relatively small aortic roots. Before closing the aortotomy after aortic valve replacement, it is advisible to move the occluder back and forth to make sure its movement and, specifically its ability to seat completely, is not limited by sutures which are too long.

Publications: Waller, B.F., Jones, M., Roberts, W.C.: Post-operative aortic regurgitation from incomplete seating of tilting-disc occluders due to overhanging knots or long sutures. Chest (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03181-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Quantification of Coronary Arterial Narrowing and of Left Ventricular Myocardial Scarring in Healed Myocardial Infarction with Chronic, Eventually Fatal, Congestive cardiac Failure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R. Virmani, Staff Fellow, PB, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A qualitative and quantitative analysis is described of the amount of ventricular wall myocardial scarring and the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major epicardial coronary arteries in 18 necropsy patients with healed transmural myocardial infarcts, and chronic, eventually fatal, congestive heart failure. In all 19 patients, the healed infarcts involved greater than 40% of the left ventricular wall, all had very dilated right and left ventricular cavities, all had hearts weighing more than 450gms (avg = 587), all had intractable congestive heart failure for longer than 3 months (avg = 2.3 years), and half had intraventricular mural thrombi. Of 1012 five-mm segments of the 4 major epicardial coronary arteries examined in the 18 patients (average 54 segments per patient), 298 segments (29%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (in 16 control subjects = 6%), 370 (37%) were 51 to 75% narrowed (controls = 35%), 227 (23%) were 26 to 50% narrowed (controls = 43%), and 117 (11%) were 1 to 25% narrowed (controls = 16%).

PHS-6040
(Rev. 10-76)

Project Description: A qualitative and quantitative analysis is described of the amount of ventricular wall myocardial scarring and the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major epicardial coronary arteries in 18 necropsy patients with healed transmural myocardial infarcts, and chronic, eventually fatal, congestive heart failure. In all 18 patients, the healed infarcts involved greater than 40% of the left ventricular wall, all had very dilated right and left ventricular cavities, all had hearts weighing more than 450gms (avg = 587), all had intractable congestive heart failure for longer than 3 months (avg = 2.3 years), and half had intraventricular mural thrombi. Of 1012 five-mm segments of the 4 major epicardial coronary arteries examined in the 18 patients (average 54 segments per patient), 298 segments (29%) were 76 to 100% narrowed in cross-sectional area by atherosclerotic plaques (in 16 control subjects = 6%), 370 (37%) were 51 to 75% narrowed (controls = 35%), 227 (23%) were 26 to 50% narrowed (controls = 43%), and 117 (11%) were 0 to 25% narrowed (controls = 16%). The amount of severe (> 75%) narrowing of the right, left anterior descending and left circumflex coronary arteries was similar in the 18 study patients. The left main coronary artery was not severely narrowed in any patient. The amount of severe narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these 3 arteries. The percent of 5-mm segments of coronary artery narrowed 76 to 100% in cross-sectional area in the 9 patients which was similar to that in the 9 patients without left ventricular aneurysm.

Publications: Virmani, R., Roberts, W.C.: Quantification of coronary arterial narrowing and of left ventricular myocardial scarring in healed myocardial infarction with chronic, eventually fatal, congestive cardiac failure. American Journal of Medicine. 68: 831-838, June 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03182-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Status of the Coronary Arteries at Necropsy in Diabetes Mellitus with Onset After Age 30 Years

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Fellow, Pathology Branch, NHLBI
Other: P. J. Palumbo, Professor of Medicine, Mayo Clinic, Rochester, MN
J. T. Lie, Professor of Pathology, Mayo Clinic, Rochester, MN
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Departments of Medicine and Pathology, Mayo Clinic, Rochester, MN

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic observations were made in 229 necropsy patients with diabetes mellitus (DM) with onset of DM after 30 years of age, 65 without (DM-CHD) and 164 with (DM+CHD) clinical evidence of coronary heart disease (CHD) and, these observations were compared to those in 183 age-sex-matched non-DM control subjects (CHD-DM) who died from a fatal coronary event. The average number of 3 major (right, left anterior descending, left circumflex) coronary arteries per patient narrowed >75 per cent in cross-sectional area by atherosclerotic plaques was similar in the 229 diabetic pateitns (DM-CHD and DM+CHD) and in the control subjects (CHD-DM), namely 2.5/3.0 and 2.5/3.0. This similarity in the amount of coronary arterial narrowing was present irrespective of the age at onset (after 30 years) or duration of DM. The DM + CHD patients had more severe narrowing of the 3 major coronary arteries than did the DM-CHD patients (p < .01).

PHS-6040
(Rev. 10-76)

Project Description: Clinical and morphologic observations were made in 229 necropsy patients with diabetes mellitus (DM) with onset of DM after 30 years of age, 65 without (DM-CHD) and 164 with (DM+CHD) clinical evidence of coronary heart disease (CHD) and, these observations were compared to those in 183 age-sex-matched non-DM control subjects (CHD-DM) who died from a fatal coronary event. The average number of 3 major (right, left anterior descending, left circumflex) coronary arteries per patient narrowed > 75 per cent in cross-sectional area by atherosclerotic plaques was similar in the 229 diabetic patients (DM-CHD and DM+CHD) and in the control subjects (CHD-DM), namely 2.5/3.0 and 2.5/3.0. This similarity in the amount of coronary arterial narrowing was present irrespective of the age at onset (after 30 years) or duration of DM. The DM + CHD patients had more severe narrowing of the 3 major coronary arteries than did the DM-CHD patients ($p < .01$). The amount of severe narrowing of the left anterior descending, left circumflex and right coronary arteries was similar in both the diabetic patients and the control subjects, and in each group, the amount of severe narrowing in the proximal halves of each of these 3 arteries was similar to that in the distal halves. The amount of severe (> 75 per cent in cross-sectional area) narrowing of the left main coronary artery was greater in the DM patients than in the non-DM controls: 13 per cent vs 6 per cent ($p < .01$). The type of treatment received by the DM patients or the adherence to the therapeutic program as measured by the level of random fasting blood sugar did not alter the amount of severe coronary narrowing observed at necropsy. Acute myocardial infarction was the most frequent fatal coronary event in the diabetic patients (55 per cent) compared to 43 per cent in the controls ($p < .01$).

Publications: Waller, B.F., Palumbo, P.J., Lie, J.T., Roberts, W.C.: Status of the coronary arteries at necropsy in diabetes mellitus with onset after age 30 years: Analysis of 229 diabetic patients with and without clinical evidence of coronary heart disease and comparison to 183 control subjects. American Journal of Medicine. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03183-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Congenital hypoplasia of portions of both right and left ventricular myocardial walls

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI

OTHER:

E. R. Smith, Professor of Cardiology, Department of Medicine, Dalhousie University, Halifax, Nova Scotia, Canada
Brian D. Blackbourne, Pathologist, District of Columbia Medical Examiner's Office, Washington, D.C.
Felix P. Arce, Pathology Department, Dalhousie University, Halifax, Nova Scotia, Canada
Nellie N. Sarkar, Pathology Deparmtent, Dalhousie University, Halifax, Nova Scotia, Canada
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Departments of Medicine and Pathology, Dalhousie University, Halifax, Nova Scotia, Canada
District of Columbia Medical Examiner's Office, Washington, D.C.

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic findings are described in 2 patients with congenital hypoplasia of portions of both right and left ventricular free walls in the absence of associated coronary or valvular heart disease. One, a 61-year-old man who had never had clinical evidence of cardiac dysfunction, died suddenly and unexpectedly. The second, a 55-year-old woman, died of progressive, eventually intractable congestive cardiac failure of 29 months duration. Although at least 22 necropsy patients have been reported previously with "parchment-like" thinning of portions of the right ventricular free wall, only one patient has been described previously with "parchment-like" thinning of portions of both right and left ventricular free walls.

PHS-6040
(Rev. 10-76)

Project Description: Clinical and morphologic findings are described in 2 patients with congenital hypoplasia of portions of both right and left ventricular free walls in the absence of associated coronary or valvular heart disease. One, a 61-year-old man who had never had clinical evidence of cardiac dysfunction, died suddenly and unexpectedly. The second, a 55-year-old woman, died of progressive, eventually intractable congestive cardiac failure of 29 months duration. Although at least 22 necropsy patients have been reported previously with "parchment-like" thinning of portions of the right ventricular free wall, only one patient has been described previously with "parchment-like" thinning of portions of both right and left ventricular free walls. The spectrum of right or right and left ventricular wall congenital hypoplasia is a broad one with nearly half of described patients dying of congestive heart failure in the first year of life and the other half reaching adulthood with or without manifestations of cardiac dysfunction.

Publications: Waller, B.F., Smith, E.R., Blackbourne, B.D., Arce, F.P., Sarkar, N.N., Roberts, W.C.: Congenital hypoplasia of portions of both right and left ventricular myocardial walls: Clinical and necropsy observations in two patients with the parchment heart syndrome. American Journal of Cardiology. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03184-01 PA

PERIOD COVERED

October 1, 1979 to September 1980

TITLE OF PROJECT (80 characters or less)

Comparison on Coronary Narrowing by Videoplanimetry and Visual Inspection

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: J. M. Isner, Staff Associate, PB, NHLBI

OTHER: M. Wu, Mathematic Statatician, Mathematical and Applied Staticical Branch, Heart and Vascular Diseases, NHLBI
R. Virmani, Staff Fellow, PB, NHLBI
A. A. Jones, Clinical Associate, NHLBI
W. C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The accuracy of determining degrees of luminal narrowing of 559 histologic sections from 61 human coronary arteries was evaluated by visual inspection under magnification (light microscopy) by three independent observers, and the results were compared to those obtained by video planimetry. With the per cent of cross-sectional area narrowing divided into four categories (0 to 25, 26 to 50, 51 to 75, and 76 to 100), both the extent of agreement between each independent observer and video planimetry, i.e., the accuracy of visual inspection under magnification and the extent of agreement among the independent observers, interobserver agreement, evaluated by the Kappa (K) statistic, were excellent.

PHS-6040
(Rev. 10-76)

Project Description: The accuracy of determining degrees of luminal narrowing of 559 histologic sections from 61 human coronary arteries was evaluated by visual inspection under magnification (light microscopy) by three independent observers, and the results were compared to those obtained by video planimetry. With the per cent of cross-sectional area narrowing divided into four categories (0 to 25, 26 to 50, 51 to 75, and 76 to 100), both the extent of agreement between each independent observer and video planimetry, i.e., the accuracy of visual inspection under magnification and the extent of agreement among the independent observers, interobserver agreement, evaluated by the Kappa (K) statistic, were excellent. With a K value of 50 per cent indicating reasonably good agreement and values of ≥70 per cent indicating strong agreement, the K values between the results of light microscopy and video planimetry by three observers were 61, 67, and 75 percent, respectively, and the extent of interobserver agreement was 72 per cent.

Publications: Isner, J.M., Wu, M., Virmani, R., Jones, A.A., and Roberts, W.C.: Comparison of degrees of coronary arterial luminal narrowing determined by visual inspection of histologic sections under magnification among three independent observers and comparison to that obtained by video planimetry. An analysis of 559 five-millimeter segments of 61 coronary arteries from eleven patients. Laboratory Investigation. 42:566-570, May 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03185-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Quantification of coronary arterial narrowing at necropsy in acute transmural myocardial infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: A. A. Jones, Clinical Associate, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We quantitatively analyzed the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire length of each of the four major coronary arteries in 27 necropsy patients with transmural acute myocardial infarction (AMI) and compared the findings with those in 22 control subjects. Of the 1403 5-mm segments examined in the 27 AMI patients, 484 (34%: controls 3%) were 76-100% narrowed in cross-sectional area by athersclerotic plaques, 528 (38%; controls 25%) were 51-75% narrowed, 319 (23%; controls 44%) were 26-50% narrowed, and only 72 segments (5%; controls 28%) were ≤ 25% narrowed.

PHS-6040
(Rev. 10-76)

Project Description: We quantitatively analyzed the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire length of each of the four major coronary arteries in 27 necropsy patients with transmural acute myocardial infarction (AMI) and compared the findings with those in 22 control subjects. Of the 1403 5-mm segments examined in the 27 AMI patients, 484 (34%; controls 3%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques, 528 (38%; controls 25%) were 51-75% narrowed, 319 (23%; controls 44%) were 26-50% narrowed, and only 72 segments (5%; controls 28%) were $\leq$ 25% narrowed. The amount of severe (> 75%) narrowing of the right, left anterior descending and left circumflex coronary arteries by atherosclerotic plaques was similar, as was the amount of severe narrowing in the distal and proximal halves of these three arteries. The number of severely narrowed 5-mm segments did not correlate significantly with the patient's age at death, the presence or absence of a history of angina pectoris or healed myocardial infarction, or with heart weight. The men, however, had a significantly greater number of severely narrowed 5-mm segments of coronary artery than the women ($p < 0.05$), and the patients with associated transmural left ventricular scars had significantly more severely narrowed segments than did patients without transmural scars.

Publications: Roberts, W.C., Jones, A.A.: Quanitfication of coronary arterial narrowing at necropsy in acute transmural myocardial infarction: Analysis and comparison of findings in 27 patients and 22 controls. Circulation. 61:786-790, April 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03186-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Pacemaker endocarditis with disseminated Petriellidium Boydii

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: W. A. Davis, Department of Medicine, Georgetown University, Washington, D.C.

OTHER: J. M. Isner, Staff Associate, PB, NHLBI
A. W. Bracey, Department of Pathology, Georgetown University, Washington, D.C.
W. C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)
Departments of Medicine and Pathology, Georgetown University, Washington, D.C.

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and morphologic findings are described in a 62-year-old woman with "mixed connective tissue disease" treated by corticosteroids who developed disseminated and fatal Petriellidium boydii infection with right-sided endocarditis. The patient was an avid gardener, the organism is ubiquitous in soil in many parts of the U.S.A. and, therefore, it is likely that the infection was introduced by this means. Endocarditis due to P. boydii has not been described previously. In the patient reported herein, massive vegetations nearly obliterated the tricuspid valve orifice, encasing a pacemaker catheter which had been inserted eight years earlier.

PHS-6040
(Rev. 10-76)

Project Description: Clinical and morphologic findings are described in a 62-year-old woman with "mixed connective tissue disease" treated by corticosteroids who developed disseminated and fatal *Petriellidium boydii* infection with right-sided endocarditis. The patient was an avid gardener, the organism is ubiquitous in soil in many parts of the U.S.A. and, therefore, it is likely that the infection was introduced by this means. Endocarditis due to *P. boydii* has not been described previously. In the patient reported herein, massive vegetations nearly obliterated the tricuspid valve orifice, encasing a pacemaker catheter which had been inserted eight years earlier. Although never previously isolated from blood cultures, *P. boydii* was isolated from 11 consecutive blood cultures. *P. boydii* is a true fungus and has only recently been appreciated as an opportunistic pathogen in a compromised host.

Publications: Davis, W.A., Isner, J.M., Bracey, A.W., Roberts, W.C., and Garagusi, V.F.: Pacemaker endocarditis with disseminated *Petriellidium Boydii*: A complication of gardening in a compromised host. Am J Medicine (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03187-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Significance of Coronary arterial Thrombus in Transmural Acute Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frank C. Brosius, III, Staff Fellow, Pathology Branch, NHLBI
Other: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In 54 necropsy patients with transmural acute myocardial infarction (AMI) and coronary arterial thrombi, histologic sections of coronary arteries containins the thrombi were examined in detail by videoplanimetry to determine 1) the amount of luminal narrowing caused by thrombi compared to that produced by underlying atherosclerotic plaques, and 2) the amount of luminal narrowing by plaques immediately proximal and distal to the thrombi. The 54 coronary arteries were narrowed by atherosclerotic plaque alone from 33-98% (mean 81) in cross-sectional area at the site of the thrombus (occlusive in 47, non-occlusive in 7); from 26-98% (mean 75) within the 2-cm segment proximal to the thrombus, and from 43-98% (mean 79) within the 2-cm segment distal to the thrombus. Of the 54 arteries, 52 (96%) were narrowed 76-98% in cross-sectional area by atherosclerotic plaque alone at or immediately proximal or distal to the thrombus and 26 (48%) of them were narrowed 91-98% by plaque alone.

PHS-6040
(Rev. 10-76)

Project Description: In 54 necropsy patients with transmural acute myocardial infarction (AMI) and coronary arterial thrombi, histologic sections of coronary arteries containing the thrombi were examined in detail by videoplanimetry to determine 1) the amount of luminal narrowing caused by thrombi compared to that produced by underlying atherosclerotic plaques, and 2) the amount of luminal narrowing by plaques immediately proximal and distal to the thrombi. The 54 coronary arteries were narrowed by atherosclerotic plaque alone from 33-98% (mean 81) in cross-sectional area at the site of the thrombus (occlusive in 47, non-occlusive in 7); from 26-98% (mean 75) within the 2-cm segment proximal to the thrombus, and from 43-98% (mean 79) within the 2-cm segment distal to the thrombus. Of the 54 arteries, 52 (96%) were narrowed 76-98% in cross-sectional area by atherosclerotic plaque alone at or immediately proximal or distal to the thrombus and 26 (48%) of them were narrowed 91-98% by plaque alone. The thrombi ranged in size from 0.1-6.0mm^2 (mean 1.4) in cross-sectional area and the underlying atherosclerotic plaque, from 3.0-21.0mm^2 (mean 8.7). Thus, among necropsy patients with transmural AMI, coronary thrombi occur at sites already severely narrowed by atherosclerotic plaques. This observation suggests a relatively minor role of coronary thrombi as a terminal event in patients with fatal AMI.

Publications: Brosius, F.C., Roberts, W.C.: Significance of coronary arterial thrombus in transmural acute myocardial infarction: Quantative analysis of degrees of narrowing by atherosclerotic plaque proximal and distal to and at the site of thrombus, area of thrombus compared to area of underlying plaque and to original area of artery, and length of thrombus from study of 54 necropsy patients. Circulation. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03188-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Coronary Artery Size in Coronary Heart Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C. S. Roberts, Summer Student, PB, NHLBI
Other: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs.	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cross-sectional area (that portion enclosed by the internal elastic membrane) of histologic sections from the first 5-mm long segments of the right, left anterior descending and left circumflex coronary arteries was determined by videoplanimetry in 98 necropsy patients with coronary heart disease and in 46 control subjects (without significant coronary narrowing). Significant ($p<0.002$) differences were observed in the mean cross-sectional area of each of the 3 major coronary arteries in the subgroups of coronary patients and among and between the control subjects and these differences resulted primarily from differences in heart weight and, to a slight extent, differences in age.

PHS-6040
(Rev. 10-76)

Project Description: The cross-sectional area (that portion enclosed by the internal elastic membrane) of histologic sections from the first 5-mm long segments of the right, left anterior descending and left circumflex coronary arteries was determined by videoplanimetry in 98 necropsy patients with coronary heart disease and in 46 control subjects (without significant coronary narrowing). Significant ($p < 0.001$) differences were observed in the mean cross-sectional area of each of the 3 major coronary arteries in the subgroups of coronary patients and among and between the control subjects and these differences resulted primarily from differences in heart weight and, to a slight extent, differences in age. Difference in sex was not significant. The 20 patients with angina pectoris had the smallest coronary arteries (mean cross-sectional area of each of the 60 arteries = $6.0mm^2$) and the smallest hearts (mean weight = 386g) and the 18 patients with healed myocardial infarcts and intractable congestive heart failure had the largest coronary arteries (mean area = $8.6mm^2$) and the largest hearts (mean weight = 588g). The 23 patients with acute transmural myocardial infarcts and the 19 with sudden coronary death had similar sized coronary arteries (mean area = $7.6mm^2$) and similar sized hearts (mean weight = 471g), and the 18 patients with healed myocardial infarcts, subsequently asymptomatic courses and non-cardiac deaths had slightly enlarged arteries (mean area = $6.9mm^2$) and hearts (mean weight = 430g). The 31 control subjects with cancer and normal or near normal sized hearts (mean weight = 309g) had the smallest coronary arteries (mean area = $5.0mm^2$) and the 16 controls with aortic-valve disease had the largest hearts (mean weight = 730g) had the largest coronary arteries (mean area = $9.6mm^2$). When heart weights were equalized (450g), older patients had larger coronary arteries than younger patients (mean area < 40 years = 6.5; 41-60 years = 6.8, and > 60 years = $7.6mm^2$).

Publications: Roberts, C.S., Roberts, W.C.: Cross-sectional area of the proximal portions of the three major epicardial coronary arteries in 98 necropsy patients with different coronary events (angina pectoris, sudden coronary death, acute myocardial infarction and healed myocardial infarction) and its relationship to heart weight, age and sex. Circulation. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03189-01 PA

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Carcinoma of the lung causing pulmonary arterial stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI

OTHER: R. D. Fletcher, Chief of Cardiology, VA Hospital, Washington, D.C.
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

VA Hospital, Washington, D.C.

LAB/BRANCH

Pathology Branch

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Although many reports have described exterior compression of one or more major extrapulmonary pulmonary arteries by neoplasm, auscultatory evidence of obstruction to one or more major pulmonary arteries has not been documented previously. In our patient, total obstruction to the left main pulmonary artery and partial obstruction to the right main pulmonary artery was documented by both angiography (during life) and necropsy, and a 33 mmHg peak systolic pressure gradient between proximal and distal right main pulmonary artery was found at catheterization.

Project Description: Although many reports have described exterior compression of one or more major extrapulmonary pulmonary arteries by neoplasm, auscultatory evidence of obstruction to one or more major pulmonary arteries has not been documented previously. In our patient, total obstruction to the left main pulmonary artery and partial obstruction to the right main pulmonary artery was documented by both angiography (during life) and necropsy, and a 33mm Hg peak systolic pressure gradient between proximal and distal right main pulmonary artery was found at catheterization. The wide splitting of the second heart sound at the cardiac base in our patient can be attributed to the peripheral pulmonary stenosis. There was not enough right ventricular failure (only a third heart sound) in the presence of the pulmonary hypertension in our patient to account for the wide splitting of the second heart sound.

Publications: Waller, B.F., Fletcher, R.D., Roberts, W.C.: Carcinoma of the lung causing pulmonary arterial stenosis. Chest. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 03190-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Comparison of Degree and Extent of Coronary Narrowing by Atherosclerotic PLague in Anterior and in Posterior Transmural Acute Myocardial Infarction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frank C. Brosius, III, Staff Fellow, Pathology Branch, NHLBI
Other: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The percent of cross-sectional area narrowing by atherosclerotic plaques alone in each five-mm long segment of each of the four major (right, left main, left anterior descending and left circumflex) epicardial coronary arteries was determined at necropsy in 50 patients with their first acute transmural myocardial infarct (MI), and the amount and extent of the coronary narrowing in the 22 patients with anterior wall MI were compared to that in the 28 patients with posterior wall MI. The percent of coronary arteries narrowed 76-100% was similar in the anterior and posterior wall MI patients (74%-vs-75%; average 3.0 of 4 coronary arteries per patient). A lower percentage of five-mm coronary segments in the anterior MI patients, however, were narrowed 76-100% than in the posterior MI patients (39%-vs-23% [p<.001]).

Project Description: The percent of cross-sectional area narrowing by atherosclerotic plaques alone in each five-mm long segment of each of the four major (right, left main, left anterior descending and left circumflex) epicardial coronary arteries was determined at necropsy in 50 patients with their first acute transmural myocardial infarct (MI), and the amount and extent of the coronary narrowing in the 22 patients with anterior wall MI were compared to that in the 28 patients with posterior wall MI. The percent of coronary arteries narrowed 76-100% was similar in the anterior and posterior wall MI patients (74%-vs-75%; average 3.0 of 4 coronary arteries per patient). A lower percentage of five-mm coronary segments in the anterior MI patients, however, were narrowed 76-100% than in the posterior MI patients (39%-vs-23% [p<.001]). Among the anterior MI patients, a higher percentage of the five-mm segments of the left anterior descending was severely (>75%) narrowed than of either posterior perfusing coronary artery. The percents of segments narrowed 76-100% for each of the major coronary arteries in the posterior MI patients, however, was similar. Thus, from the standpoint of the coronary artery, our necropsy patients with posterior wall MI had more extensive and severe narrowing than did our patients with anterior wall MI. If the coronary arteries had not been examined in a quantitative manner, this difference in severity would not have been apparent.

Publication: Brosius, F.C. III, Roberts, W.C.: Comparison of Degree and Extent of Coronary Narrowing by Atherosclerotic Plaque in Anterior and in Posterior Transmural Acute Myocardial Infarction. Analysis of 200 major epicardial coronary areteries and of 2586 five-mm coronary segments in 50 necropsy patients with fatal first infarct. Circulation (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03191-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Status of the Coronary Arteries at Necropsy in Sudden Coronary Death

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI
Other: A. A. Jones, Clinical Associate, NHLBI
Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A qualitative and quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major coronary arteries in 31 patients dying suddenly from coronary heart disease ("sudden coronary death") is described at necropsy. A total of 1564 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries were examined in 31 patients with sudden coronary death and the observations in them were compared to those made from examination of 1100 five-mm segments of major epicardial coronary artery in 25 control subjects. Of the 1564 five-mm segments examined in the 31 study patients, 557 (36%) were 76-100% narrowed in cross-sectional area by athersclerotic plaques (controls = 3%); 536 (34%) were 51-75% narrowed (controls = 22%), 360 (23%) were 26-50% (controls = 42%) and only 111 segments (7%) were ≤ 25% narrowed (controls = 33%).

PHS-6040
(Rev. 10-76)

Project Description: A qualitative and quantitative analysis of the degree and extent of coronary arterial narrowing by atherosclerotic plaques in the entire lengths of each of the 4 major coronary arteries in 31 patients dying suddenly from coronary heart disease ("sudden coronary death") is described at necropsy. A total of 1564 five-mm long segments of the left main, left anterior descending, left circumflex and right coronary arteries were examined in 31 patients with sudden coronary death and the observations in them were compared to those made from examination of 1100 five-mm segments of major epiardial coronary artery in 25 control subjects. An average of 25 cm (50 five-mm segments) of coronary artery were examined from each patient and an average of 22 cm (44 five-mm segments) from each control subject. Of the 1564 five-mm segments examined in the 31 study patients, 557 (36%) were 76-100% narrowed in cross-sectional area by atherosclerotic plaques (controls = 3%); 536 (34%) were 51-75% narrowed (controls = 22%), 360 (23%) were 26-50% (controls = 42%) and only 111 segments (7%) were $\leq$ 25% narrowed (controls = 33%). The amount of severe (>75%) narrowing of the right, left anterior descending and left circumflex coronary arteries was similar. The amount of severe (>75%) narrowing in the distal one-half of the right, left anterior descending and left circumflex coronary arteries was similar to that in the proximal halves of these 3 arteries. The number of 5-mm coronary segments narrowed 76 to 100% in cross-sectional area in the 31 study patients was not affected by the patient's age at death, sex, presence or absence of previous angina pectoris or myocardial infarction, or the weight of the heart.

Publication: Roberts, W.C., Jones, A.A., Waller, B.F.: Status of the Coronary Arteries at Necropsy in Sudden Coronary Death: A Qualitative and Quantitative Analysis of the Degrees of Luminal Narrowing. Second US-USSR Joint Symposium on Sudden Death.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03192-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Sudden death while running in conditioned runners aged 40 years or over

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Clinical and necropsy observations are described in 5 runners (aged 40-54 years, all men) who ran 14-110 miles/week (mean = 52) for 1-10 years (mean = 4). None had clinical evidence of cardiac disease before they became habitual runners. All died while running, and at necropsy all had severe atherosclerotic coronary luminal narrowing. Of the 5 men, at least 4 had hypercholesterolemia; 2 had systemic hypertension; 1 had angina pectoris and none had clinical evidence of an acute myocardial infarct. The single symptomatic runner also had an abnormal resting electrocardiogram and a positive exercise stress test. The electrocardiogram (4 patients) and exercise stress tests (3 patients) in the other men were negative. At autopsy, all 5 had >75% cross-sectional area narrowing by atherosclerotic plaques of the right, left anterior descending and left circumflex coronary arteries.

875

PHS-6040
(Rev. 10-76)

Project Description: Clinical and necropsy observations are described in 6 runners who ran 14-110 miles/week (mean = 52) for 1-10 years (mean = 4). None had clinical evidence of cardiac disease before they became habitual runners. All died while running, and at necropsy all had coronary disease: severe atherosclerotic luminal narrowing in 5 (aged 40-53 years, all white men) and a congenital coronary anomaly without atherosclerosis in 1 (a 17-year-old black girl). Of the 5 men, at least 4 had hypercholesterolemia; 2 had systemic hypertension; 1 had angina pectoris and none had clinical evidence of an acute myocardial infarct. The single symptomatic runner also had an abnormal resting electrocardiogram and a positive exercise stress test. The electrocardiogram (4 patients) and exercise stress tests (3 patients) in the other men were negative. At autopsy, all 5 men had greater than 75% cross-sectional area narrowing by atherosclerotic plaques of the right, left anterior descending and left circumflex coronary arteries. Of the 3 men in whom the entire lengths of these 3 coronary arteries and also the left main were examined histologically (total 5-mm segments = 153), 73 (48%) were narrowed greater than 75% in cross-sectional area by atherosclerotic plaques and 32 (21%), 51-75%. Four of the 5 men had healed (clinically silent) myocardial infarcts. Thus, coronary disease appears to be the major killer of conditioned runners.

Publications: Waller, B.F., Roberts, W.C.: Sudden death while running in conditioned runners aged 40 years or over. American Journal of Cardiology. 45:1292-1300, June 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03193-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Radiation Heart Disease.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Frank C. Brosius, III, Staff Fellow, Pathology Branch, NHLBI

OTHER: Bruce F. Waller, Staff Associate, Pathology Branch, NHLBI
William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Certain clinical and necropsy findings are described in 16 young (aged 15 to 33 years) patients who received >3500 rad to the heart five to 144 months before death. All 16 had some radiation-induced damage to the heart: 15 had thickened pericardia (five of whom had evidence of cardiac tamponade); eight had increased interstitial myocardial fibrosis, particularly in the right ventricle; 12 had fibrous thickening of the mural endocardium and 13, of the valvular endocardium. Except for valvular thickening, the changes were more frequent in the right than in the left side of the heart, presumably because of higher radiation doses to the anterior cardiac surface. Six of the 16 study patients and one of 10 control subjects had one or more major epicardial coronary arteries narrowed 76-100 per cent in cross-sectional area by atherosclerotic plaque.

PHS-6040
(Rev. 10-76)

Project Description: Certain clinical and necropsy findings are described in 16 young (aged 15 to 33 years) patients who received >3500 rads to the heart five to 144 months before death. All 16 had some radiation-induced damage to the heart: 15 had thickened pericardia (five of whom had evidence of cardiac tamponade); eight had increased interstitial myocardial fibrosis, particularly in the right ventricle; 12 had fibrous thickening of the mural endocardium and 13, of the valvular endocardium. Except for valvular thickening, the changes were more frequent in the right than in the left side of the heart, presumably because of higher radiation doses to the anterior cardiac surface. Six of the 16 study patients and one of 10 control subjects had one or more major epicardial coronary arteries narrowed 76-100 per cent in cross-sectional area by atherosclerotic plaque; one patient had a healed myocardial infarct at necropsy and one died suddenly. In 10 patients and in the 10 controls, the four major epicardial coronary arteries were examined quantitatively: 6 per cent of the 469 5-mm segments of coronary artery from the patients were narrowed 76-100 per cent (controls = 0.2 per cent, p = .06) and 22 per cent were narrowed 51-75 per cent (controls = 12 per cent). The proximal portion of the arteries in the patients had significantly more narrowing than the distal portions. The arterial plaques in the patients were largely composed of fibrous tissue; the media were frequently replaced by fibrous tissue, and the adventitia were often densely thickened by fibrous tissue. In five patients, there was focal thickening (with or without luminal narrowing) of the intramural coronary arteries. Thus, radiation to the heart may produce a wide spectrum of functional and anatomical changes, but particularly damage to the pericardia and the underlying epicardial coronary arteries.

Publications: Brosius, F.C. III, Waller, B.F., Roberts, W.C.: Radiation Heart Disease. Analysis of 16 young (aged 15 to 33 years) necropsy patients who received over 3500 rads to the heart. American Journal of Medicine (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03194-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Coronary Arterial Disease in Systemic Lupus Erythematosus

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Yasmeen S. Haider, Staff Fellow, Pathology Branch, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The degrees of cross-sectional area luminal narrowing by atherosclerotic plaques of each five-mm long segment of each of the four major (right, left main, left anterior descending and left circumflex) epicardial coronary arteries in 22 necropsy patients (age 16-37 years, 21 women) with systemic lupus erythematosus (SLE) was determined and the findings compared to that in 13 control subjects. Of 623 five-mm coronary segments in the SLE patients, 80 (13%) were narrowed 76-100% (controls = 0 of 431 segments); 125 (20%), 51-75% (controls = 6%); 273 (44%), 26-50% (controls = 63%) and 145 (23%), 0-25 (controls = 31%).

PHS-6040
(Rev. 10-76)

Project Description: The degrees of cross-sectional area luminal narrowing by atherosclerotic plaques of each five-mm long segment of each of the four major (right, left main, left anterior descending and left circumflex) epicardial coronary arteries in 22 necropsy patients (age 16-37 years, 21 women) with systemic lupus erythematosus (SLE) was determined and the findings compared to that in 13 control subjects. Of 623 five-mm coronary segments in the SLE patients, 80 (13%) were narrowed 76-100% (controls = 0 of 431 segments); 125 (20%), 51-75% (controls = 6%); 273 (44%), 26-50% (controls = 63%) and 145 (23%), 0-25 (controls = 31%). The 22 SLE patients consisted of 10 patients in whom one or more of the four major coronary arteries were narrowed 76-100% in cross-sectional area and of 12 patients who had lesser degrees of narrowing. The latter 12 patients had similar degrees of coronary narrowing to that in the 13 control subjects. The 10 SLE patients with compared to the 12 SLE patients without severe (>75%) coronary narrowing had significantly higher mean values of total serum cholesterol (382 - vs - 290 mg/dl), higher mean systolic/diastolic arterial pressures (175/119 - vs - 151/93 mm Hg), a higher frequency of mitral valvular disease (seven of 10 patients - vs - 0 of 12 patients), and a higher frequency of pericardial adhesions (seven of 10 patients - vs - three of 12 patients).

Publication: Haider, Y.S., Roberts, W.C.: Coronary Arterial Disease in Systemic Lupus Erythematosus: Quantification of degrees of narrowing in 22 necropsy patients (21 women) aged 16 to 37 years. American Journal of Medicine (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03195-01 PA

PERIOD COVERED
October 1, 1979 to Setember 30, 1980

TITLE OF PROJECT (80 characters or less)
Quantification of Amounts of Coronary Arterial Narrowing in Patients with Types II and IV Hyperlipoproteinemia and in Those with Known Normal Lipoprotein Patterns

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Henry S. Cabin, Clinical Associate, NHLBI

OTHER: William C. Roberts, Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The amount of cross-sectional area narrowing by atherosclerotic plaques in each 5-mm long segment of the left main, left anterior descending, left circumflex and right coronary arteries was analyzed at necropsy in 15 patients with type II hyperlipoproteinemia (HLP), in 13 with type IV HLP and in 10 with known normal lipoprotein patterns. All 38 study patients had clinical evidence of coronary heart disease. Of the 2593 five-mm segments examined histologically, the percents narrowed in 4 categories of cross-sectional area narrowing were as follows: 76-100%: type II = 39, type IV = 67, normal = 35 (controls = 4); 51-75%: type II = 36, type IV = 20, normal = 38 (controls = 33); 26-50%: type II = 16, type IV = 10, normal = 16 (controls = 50); 0-25%: type II = 9, type IV = 3, normal = 11 (controls = 13).

Project Description: The amount of cross-sectional area narrowing by atherosclerotic plaques in each 5-mm long segment of the left main, left anterior descending, left circumflex and right coronary arteries was analyzed at necropsy in 15 patients with type II hyperlipoproteinemia (HLP), in 13 with type IV HLP and in 10 with known normal lipoprotein patterns. All 38 study patients had clinical evidence of coronary heart disease. Of the 2593 five-mm segments examined histologically, the percents narrowed in 4 categories of cross-sectional area narrowing were as follows: 76-100%: type II = 39, type IV = 67, normal = 35 (controls = 4); 51-75%: type II = 36, type IV = 20, normal = 38 (controls = 33); 26-50%: type II = 16, type IV = 10, normal = 16 (controls = 50); 0-25%: type II = 9, type IV = 3, normal = 11 (controls = 13). Utilizing a scoring system of 1 to 4 for the 4 categories of narrowing, the mean scores per 5-mm segment for the patients with type II HLP and for those with normal lipoprotein patterns were identical (3.0) and significantly less than for the patients with type IV HLP (3.5); the mean scores per 5-mm segment for all 3 study groups were significantly greater than for the control subjects (2.3), but none of the latter had symptomatic coronary heart disease. Thus, our patients with type IV HLP had significantly more severe coronary narrowing than did our patients with type II HLP or those with normal lipoprotein patterns. Both latter groups had similar degrees of narrowing.

Publication: Cabin, H.S., Roberts, W.C.: Quantification of Amounts of Coronary Arterial Narrowing in Patients with Types II and IV Hyperlipoproteinemia and in Those with Known Normal Lipoprotein Patterns. A comparative histologic analysis of 1952 five-mm segments of coronary artery in 38 necropsy patients with symptomatic myocardial ischemia and of 641 five-mm segments in 15 control subjects. Annals Internal Medicine (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03196-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Bicuspid Aortic Valve With Regurgitation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: William C. Roberts, Chief, Pathology Branch, NHLBI

OTHER: Andrew G. Morrow, Surgery Branch, NHLBI
Charles L. McIntosh, Surgery Branch, NHLBI
Michael Jones, Surgery Branch, NHLBI
Stephen E. Epstein, Cardiology Branch, NHLBI

COOPERATING UNITS (if any)

Surgery and Cardiology Branches, NHLBI

LAB/BRANCH
Pathology Branch

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MAN YEARS:	PROFESSIONAL:	OTHER:
416 hrs.	416 hrs.	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Although stenosis and infective endocarditis are commonly appreciated complications of the congenitally bicuspid aortic valve, pure, severe aortic regurgitation complicating this congenital malformation, unassociated with either stenosis or infection, is not well recognized. Among 189 patients who had aortic valve replacement at the National Heart Institute because of isolated, pure aortic regurgitation, the congenitally bicuspid aortic valve, never the site of infective endocarditis, was responsible for the aortic regurgitation in 13 (7%). This report described certain clinical and morphologic findings in 13 men, aged 26 to 65 years (mean 43), who required aortic-valve replacement because of severe aortic regurgitation secondary to non-infective, non-stenotic congenitally bicuspid aortic valves.

Project Description: Although stenosis and infective endocarditis are commonly appreciated complications of the congenitally bicuspid aortic valve, pure, severe aortic regurgitation complicating this congenital malformation, unassociated with either stenosis or infection, is not well recognized. Among 189 patients who had aortic valve replacement at the National Heart Institute because of isolated, pure aortic regurgitation, the congenitally bicuspid aortic valve, never the site of infective endocarditis, was responsible for the aortic regurgitation in 13 (7%). This report described certain clinical and morphologic findings in 13 men, aged 26 to 65 years (mean 43), who required aortic-valve replacement because of severe aortic regurgitation secondary to non-infective, non-stenotic congenitally bicuspid aortic valves. Although not generally recognized, the non-infected congenitally bicuspid aortic valve is an important cause of pure aortic regurgitation severe enough to warrant aortic=valve replacement.

Publication: Roberts, W.C., Morrow, A.G., McIntosh, C.L., Jones, M., and Epstein, S.E.: Congenitally Biscuspid Aortic Valve Causing Severe, Pure Aortic Regurgitation Without Superimposed Infective Endocarditis: An Analysis of 13 Patients Requiring Aortic Valve Replacement. CIRCULATION (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03197-01 PA

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Vascular Structure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Others: None

COOPERATING UNITS (if any)

None

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs.	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A review of the ultrastructure of normal and diseased blood vessels.

PHS-6040
(Rev. 10-76)

Project number Z01 HL 03197-01 PA

Project Description: A review was made of the ultrastructure of blood vessels, including aorta, elastic and muscular arteries, arterioles, capillaries, venules and veins, with emphasis on their individual cellular and extracellular components as seen first in the normal state and then in atherosclerosis.

Publications: Ferrans, V. J.: Vascular structure. In Patel, D. J. and Vaishnav, R. N. (Eds.): Basic Hemodynamics and Its Role in Disease Processes. Baltimore, University Park Press, 1980, Chapter 3, pp. 105-154.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03198-01 PA

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effects of Vitamin E and Selenium on Adriamycin Toxicity in Dogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	John Van Vleet, Professor of Pathology, School of Veterinary Medicine, Purdue University		
	W. E. Weirich, School of Veterinary Medicine, Purdue University		

COOPERATING UNITS (if any)

Purdue University School of Veterinary Medicine

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Administration of vitamin E (as α-tocopherol acetate) and selenium (as sodium selenite) failed to provide adequate protection against the development of adriamycin-induced chronic cardiotoxicity in dogs.

887

Project Description: Chronic adriamycin intoxication was produced in 3 groups of Beagle dogs by weekly intravenous injections (1 mg/kg body weight) for 20 weeks (cumulative dose 400 mg/m^2). Group A (6 dogs) received adriamycin only; Group B (6 dogs) was given adriamycin and weekly doses of vitamin E (17 mg/kg body weight) as α-tocopherol acetate, and Group C (6 dogs) received adriamycin and weekly doses of vitamin E as in Group B and selenium (0.06 mg/kg body weight as selenite). Each of the 18 dogs developed cardiomyopathy and death occurred in 11 dogs during weeks 17-20. Mortality was lowest in Group B (2 of 6) but no differences between groups were seen either in survival time of the dogs that died or in severity of cardiomyopathy. Congestive heart failure with transudation was present in 4 of 11 dogs that died. Cardiac histopathology was characterized by vacuolar degeneration of myocytes. Myocardial damage was most severe in left ventricle and ventricular septum, intermediate in right ventricle and left atrium and least in right atrium. Ultrastructural study demonstrated that an early alteration in damaged myocytes was distention of sarcoplasmic reticulum to form sarcoplasmic vacuoles. Occasional damaged fibers had myofibrillar lysis and focal proliferation of sarcoplasmic reticulum.

This study demonstrates that the dog offers a suitable model for studies of chronic adriamycin cardiotoxicity in man. Lack of cardioprotection from vitamin E and selenium supplementation fails to support the proposed role of lipoperoxidative damage in the development of chronic adriamycin cardiomyopathy.

Publications: Van Vleet, J. F., Ferrans, V. J., and Weirich, W. E.: Cardiac disease induced by chronic adriamycin administration in dogs and an evaluation of vitamin E and selenium as cardioprotectants. Am. J. Pathol. 99: 13-42, 1980.

Van Vleet, J. F., and Ferrans, V. J.: Cutaneous lesions and hematologic alterations in chronic adriamycin intoxication in dogs with and without vitamin E and selenium supplementation. Am. J. Vet. Res. 41: 691-699, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03199-01 PA

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cardiac Structural Changes Produced by Defibrillator Shocks

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Others: John Van Vleet, Professor of Pathology, School of Veterinary Medicine, Purdue University
W. A. Tacker, Jr., Biomedical Engineering Center, Purdue University
L. A. Geddes, Biomedical Engineering Center, Purdue University

COOPERATING UNITS (if any)
Purdue University

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The cardiac lesions produced in dogs by single transthoracic damped sinusoidal waveform shocks consisted of focal areas of myocyte necrosis with contraction bands and calcific deposits. Inflammatory infiltrates consisted first of neutrophilic leukocytes and then of macrophages. These lesions underwent gradual resolution over a period of 8 weeks.

PHS-6040
(Rev. 10-76)

Project Description: Fifty-six dogs were allotted to eight groups and were given no shock or a single transthoracic damped sinusoidal waveform shock of 1, 3, 6, 9, 12, 15, and 20 A/kg of body weight. The dogs were euthanatized at 1 day, 2 days, 4 days, 2 weeks, and 8 weeks after shocking and small blocks of ventricular myocardium were collected for ultrastructural study from dogs given large (15 and 20 A/kg) shocks from the center of the defibrillator-induced areas of myocardial necrosis. Early (days 1, 2, and 4 after shock) ultrastructural alterations in myocardium were characterized by myocyte necrosis with contraction bands (myofibrillar degeneration). Mitochondria in damaged myocytes had flocculent matrical densities at 1 day after shock; by 2 days or more after shock mitochondria were mineralized with dense granular and spicular matrical deposits. Mineralization was also present in the lysed masses of contractile material in necrotic myocytes by 2 days or more after shock. Interstitial edema was prominent for the first 4 days after shock. Numerous neutrophilic granulocytes were in the interstitium and had invaded necrotic myocytes at 1 and 2 days after shock. By 4 days, many macrophages were infiltrated in the interstitium and into necrotic fibers and had engulfed masses of necrotic, and often mineralized sarcoplasmic debris. External laminae persisted in myocytes undergoing necrosis.

Resolving lesions were present in areas of damaged myocardium at 2 weeks and 8 weeks after shock. Only remnants of external laminae remained where myocytes had undergone necrosis. The interstitium contained active-appearing fibroblasts, abundant accumulations of collagen fibrils, and occasional macrophages. In these areas of myocardial scarring, some myocytes had persisted but were degenerated as indicated by atrophy, lysis of myofibrils (myocytolysis), and focal proliferation of elements of sarcoplasmic reticulum.

Publications: Van Vleet, J. F., Tacker, W. A. Jr., Geddes, L. A., and Ferrans, V. J.: Sequential ultrastructural alterations in ventricular myocardium of dogs given large single transthoracic damped sinusoidal waveform defibrillator shocks. Am. J. Vet. Res. 41: 493-501, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03200-01 PA

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cardiac Ultrastructure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief Ultrastructure Section PA NHLBI

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A comprehensive review of the applications of electron microscopy of the human heart to diagnostic pathology.

891

PHS-6040
(Rev. 10-76)

Project number Z01 HL 03200-01 PA

Project Description: The applications of electron microscopy of the heart are discussed under the following categories:

1. Normal myocardial ultrastructure.
2. Myocardial hypertrophy.
3. The cardiomyopathies.
4. Myocardial ischemia.
5. Myocarditis.
6. Acute rheumatic fever and chronic rheumatic valvular disease.
7. Other diseases of cardiac valves and mural endocardium.
8. Cardiac tumors.

Publications: Ferrans, V. J.: The Heart. In Johannessen, J. V. (Ed.): Electron Microscopy in Human Medicine. New York, McGraw-Hill, 1979, Volume No. 5. (In press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03701-01 PA

PERIOD COVERED

October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Effect of Vitamin E and Selenium on Adriamycin Toxicity in Rabbits

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Others: John Van Vleet, Professor of Pathology, School of Veterinary Medicine, Purdue University

COOPERATING UNITS (if any)

Purdue University, School of Veterinary Medicine

LAB/BRANCH

Pathology Branch

SECTION

Ultrastructure Section

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 416 hrs	PROFESSIONAL: 416 hrs	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The possible protective effects of vitamin E and selenium against adriamycin-induced cardiotoxicity were investigated using the rabbit model system. Cardioprotection by vitamin E and/or selenium was found to be very limited, and only supplements of large amounts of vitamin E provided even partial protection.

Project Description: Chronic adriamycin intoxication was produced in 50 weanling rabbits by weekly injections of 2.4 mg ADR/kg of body weight for up to 17 weeks. An evaluation was made of the protective value of weekly injected supplements, given 24 hours before each adriamycin injection, of: 1) low vitamin E (α-tocopherol acetate, 17 mg/kg), 2) high vitamin E (α-tocopherol acetate, 170 mg/kg), 3) selenium (as sodium selenite, 0.06 mg/kg) and 4) combined low vitamin E and selenium. The incidence of cardiomyopathy was high in both supplemented and unsupplemented rabbits. Mean cardiomyopathy scores were low in unsupplemented rabbits and moderately high in the supplemented rabbits (which survived longer), but were lower in rabbits given high vitamin E than in the other supplemented groups. Supplements of vitamin E, selenium, or both, prolonged survival of animals treated chronically with adriamycin, but only supplements of large amounts of vitamin E provided any evidence of even partial protection against chronic cardiotoxicity.

Publications: Van Vleet, J. F., and Ferrans, V. J.: Evaluation of vitamin E and selenium protection against chronic adriamycin toxicosis in rabbits. Cancer Treat. Rep. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03702-01 PA

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Calcific Deposits in Porcine Valvular Bioprostheses

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
Others:	Steven W. Boyce, Ultrastructure Section	PA	NHLBI
	Margaret E. Billingham, Asst. Prof., Department of Pathology, Stanford Univ. School of Medicine		
	Michael Jones, Senior Surgeon, Surgery Branch		NHLBI
	Tokuhiro Ishihara, Ultrastructure Section	PA	NHLBI

COOPERATING UNITS (if any)

Department of Pathology, Stanford University School of Medicine, Stanford, CA
Surgery Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A detailed description is given of the structure and pathogenesis of calcific deposits that develop in porcine bioprostheses implanted as substitute cardiac valves in patients. The two main sites of localization of these deposits are surface thrombi and the connective tissue in the cusps.

PHS-6040
(Rev. 10-76)

Project Description: Gross, histologic and ultrastructural studies were made of 14 porcine valvular bioprostheses that were found to contain calcific deposits at the time of removal either at reoperation (13 patients) or necropsy (1 patient). Eleven bioprostheses had been in the mitral position, 1 in the aortic, 1 in the tricuspid, and 1 in a pulmonic conduit. The ages of the patients at the time of implantation ranged from 2.5 to 65 years (average = 32), and the bioprostheses had been in place from 3 to 94 months (average = 39). From analysis of these 14 bioprostheses and from review of reports concerning 37 other calcified porcine bioprostheses, the following conclusions appear justified:

1. Calcific deposits occur commonly in bioprostheses implanted in patients of all ages, but are more likely to become severe and clinically significant in children and in young adults than in older patients.

2. Calcific deposits can lead to prosthetic valvular stenosis, because they can limit the mobility of the cusps; however, they also can be associated with prosthetic valvular regurgitation.

3. Metabolic disorders that could contribute to bioprosthetic calcification are not identifiable in most patients having calcified prosthetic tissue valves.

4. The two main sites of deposition of calcium phosphate in porcine valvular bioprostheses are the connective tissue in the cusps, particularly in the spongiosa, and small thrombi on the surfaces.

5. Calcification of connective tissue first involves the collagen fibrils; calcification of thrombi, the mitochondria in platelets and leukocytes trapped in the mesh of fibrin. From these two sites, calcific deposits can grow and spread into other areas of the cusps.

Publications: Ferrans, V. J., Boyce, S. W., Billingham, M. E., Jones, M., Ishihara, T. and Roberts, W. C.: Calcific deposits in porcine bioprostheses: Structure and pathogenesis. Am. J. Cardiol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03703-01 PA

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Structure of Human Pericardium

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Tokuhiro Ishihara, Guest Worker, Ultrastructure Section	PA	NHLBI
Others:	Victor J. Ferrans, Chief, Ultrastructure Section	PA	NHLBI
	S. W. Boyce, Ultrastructure Section	PA	NHLBI
	Oichi Kawanami, Visiting Expert, Ultrastructure Section,	PA	NHLBI
	Michael Jones, Senior Surgeon, Surgery Branch	PA	NHLBI
	W. C. Roberts, Chief, Pathology Branch	PA	NHLBI

COOPERATING UNITS (if any)

Surgery Branch

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A detailed description of the ultrastructure of human pericardium.

PHS-6040
(Rev. 10-76)

Project Description: Histologic and ultrastructural studies were made of normal anterior parietal pericardium from 7 patients. Parietal pericardium is composed of 3 layers: 1) the serosa, which consists of a surface layer of mesothelial cells and a narrow submesothelial space; 2) the fibrosa, which contains variously oriented layers of collagen fibrils and small elastic fibers, and 3) the epipericardial connective tissue layer, which mainly contains large, coarse bundles of collagen and forms part of the pericardiosternal ligament. Scanning electron microscopic examination is most useful for study of the surface features of pericardial mesothelial cells, which have single cilia and are covered with microvilli. The latter bear friction and increase the surface area for fluid transport. Junctional complexes between adjacent mesothelial cells consist of desmosomes, which reinforce intercellular adhesion, and zonulae occludentes, which form permeability barriers. Actin-like (50 Å in diameter) filaments are present in microvilli and immediately subjacent regions of the cells; these filaments mediate changes in cell shape. Intermediate (100 Å in diameter) filaments are associated with desmosomes and form bundles in the perinuclear regions; these filaments provide structural support to the cytoplasm. The collagen in the fibrosa has a wavy or "crimped" configuration, which permits stretching of the pericardial connective tissue.

Publications: Ishihara, T., Ferrans, V. J., Jones, M., Boyce, S. W., Kawanami, O., and Roberts, W. C.: Histologic and ultrastructural features of normal human parietal pericardium. Am. J. Cardiol. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03704-01 PA

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Subplasmalemmal Linear Densities in Mononuclear Cells in Lung

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Oichi Kawanami	Visiting Expert, Ultrastructure Section	PA	NHLBI
Others:	Victor J. Ferrans	Chief, Ultrastructure Section	PA	NHLBI
	R. G. Crystal	Chief, Pulmonary Branch		NHLBI

COOPERATING UNITS (if any)

Pulmonary Branch, NHLBI

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Subplasmalemmal linear densities, consisting of a thin layer of electron-dense material immediately subjacent to the inner aspect of the plasma membrane, were found in cells of the mononuclear phagocyte system in lung biopsies from patients with fibrotic disorders. Two types of subplasmalemmal linear densities were found: impaired and paired. The latter formed junctions that differed morphologically from other types of junctions. Such junctions may contribute to the aggregation and immobilization of mononuclear phagocytes.

PHS-6040
(Rev. 10-76)

Project number Z01 HL 03704-01 PA

Project Description: The presence of subplasmalemmal linear densities in cells of the mononuclear phagocyte system was investigated in pulmonary biopsies from 33 patients with fibrotic lung disorders. Subplasmalemmal linear densities, consisting of a thin layer of electron-dense material immediately subjacent to the inner leaflet of the plasma membrane, were found in 30 of the 33 patients, including each of 6 patients with pulmonary sarcoidosis, 18 of 19 patients with idiopathic pulmonary fibrosis, 4 of 5 patients with collagen-vascular diseases, 1 patient with pulmonary lymphangioleiomyomatosis, and 1 patient with marked interstitial pulmonary fibrosis associated with squamous cell carcinoma of the lung. Subplasmalemmal linear densities were found in epithelioid cells, macrophages and giant cells in granulomas in the 6 patients with sarcoidosis, and in alveolar macrophages in 4 of these patients. In patients with other fibrotic lung disorders, subplasmalemmal linear densities were limited in distribution to interstitial and alveolar macrophages. In all patients with sarcoidosis some of the subplasmalemmal linear densities of adjacent mononuclear phagocytes, particularly of those in granulomas, were paired and formed specialized intercellular junctions. Such junctions also were observed in macrophages in 10 of the patients with other fibrotic lung disorders. The junctions formed by subplasmalemmal linear densities differed from other types of junctional structures. Subplasmalemmal linear densities appear to function in: 1) the binding of actin filaments to the cytoplasmic surface of the plasma membrane, and 2) the formation of intercellular junctions, which may contribute to the immobilization of mononuclear phagocytes in granulomas and alveolar lumina.

Publications: Kawanami, O., Ferrans, V. J., and Crystal, R. G.: Subplasmalemmal linear densities in cells of the mononuclear phagocyte system in lung. Am. J. Pathol. 100: 131-150, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 03705-01 PA

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Adriamycin Toxicity in Pigs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Victor J. Ferrans, Chief, Ultrastructure Section PA NHLBI
Others: John F. Van Vleet, Professor of Pathology, School of Vet. Med., Purdue Univ.
Linda A. Greenwood, School of Vet. Med., Purdue Univ.

COOPERATING UNITS (if any)

School of Veterinary Medicine, Purdue University, West Lafayette, IN

LAB/BRANCH
Pathology Branch

SECTION
Ultrastructure Section

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
416 hrs	416 hrs	

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The toxicity of therapeutic doses of adriamycin was evaluated in pigs. An animal model was developed for the study of the cardiac chronic lesions produced by adriamycin. This represents the only large animal model system available for this purpose.

901

PHS-6040
(Rev. 10-76)

Project Description: In ten experiments, 53 castrated male 4- to 8-week-old weanling pigs were given adriamycin in doses of 0.64, 1.0, 1.6, 3.2, or 6.4 mg/kg/week for up to 20 weeks. Survival time was prolonged in younger pigs and in pigs given smaller but more frequent dosages of adriamycin. Characteristic gross and histopathologic alterations of adriamycin toxicosis were observed in pigs given 1.0, 1.6, 3.2, or 6.4 mg/kg/week mean dosages. The most frequent lesions were in the alimentary tract, myeloid and lymphoid tissues, skin, and perivascular tissues at injection sites. Alimentary tract lesions consisted of mucosal epithelial atrophy, with secondary fibrinonecrotic inflammation in the oral cavity and large intestine. Marked hypoplasia was seen in bone marrow and lymphoid tissues, with frequent terminal hemorrhagic diathesis and septicemia. Several days before death, the pigs developed severe dermatitis over the ventral portion of the abdomen and inner surfaces of the limbs. Perivascular necrosis and cellulitis produced by extravasation of adriamycin was a frequent complication of treatment. Terminal severe acute pneumonia occurred in most pigs.

Pericarditis or cardiomyopathy (or both) developed in 14 pigs given 0.64, 1.0, or 1.6 mg/kg each week (mean cumulative dosage 520.5 mg/m^2 of body surface). Characteristic histopathologic and ultrastructural alterations in affected cardiac muscle cells were vacuolar degeneration, myocytolysis, and hyaline necrosis. Renal lesions also developed in pigs with chronic adriamycin toxicosis. Systemic antibiotic treatment did not prolong survival of adriamycin-treated pigs in two experiments, but did in one other experiment. In conclusion, the pig represents a useful large animal model for the study of chronic adriamycin cardiotoxicity.

Publications: Van Vleet, J. F., Greenwood, L. A., and Ferrans, V. J.: Pathologic features of adriamycin toxicosis in young pigs: nonskeletal lesions. Am. J. Vet. Res. 40: 1537-1552, 1979.

ANNUAL REPORT OF THE
CLINIC OF SURGERY
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1979 through September 30, 1980

The clinical and laboratory programs of the Surgery Branch have, as in past years, largely centered upon the study of operative methods for the correction of congenital and acquired heart and lung diseases, assessment of the results of such operations, and laboratory studies related to cardiovascular physiology and pharmacology.

A long-term and continuing interest of the Surgery Branch has been the evaluation of the role of operation in patients with hypertrophic cardiomyopathy. As of this time 300 patients with hypertrophic cardiomyopathy have been operated upon. In general, operation has only been recommended to severely symptomatic patients who have not benefitted from treatment with Inderal or Verapamil. Recently, however, operations have been recommended and carried out in an unusual subgroup of patients with this disease.

Left Ventricular Myotomy and Myectomy in Patients with Obstructive Hypertrophic Cardiomyopathy and Previous Cardiac Arrest. Left ventricular myotomy and myectomy was carried out in 15 patients with obstructive hypertrophic cardiomyopathy solely because of a previously documented episode of cardiac arrest. Prior to arrest, each patient had either no or only minimal functional limitation, and, therefore, would not have met the usual criteria for operation: severe symptoms unresponsive to medical therapy. Of the 15 patients, one died in the perioperative period, and one died suddenly and unexpectedly nine months postoperatively. The remaining patients have survived one to six years after operation; all but one of the survivors is asymptomatic, and this one has only mild symptoms. Operation resulted in marked decrease or abolition of the left ventricular outflow gradient under basal conditions in the seven patients who have been studied postoperatively. Significant residual outflow obstruction was demonstrated after operation in the patient who later died.

Sudden death in patients with obstructive hypertrophic cardiomyopathy appears usually to result from ventricular arrhythmia, and prevention of recurrent fatal arrhythmia is the goal of treatment in patients who have had cardiac arrest and been resuscitated. Such patients should be treated indefinitely with antiarrhythmic drugs. In addition, when severe outflow obstruction is present, we postulate that effective operative relief of obstruction and consequent lowering of left ventricular systolic pressure will provide additional protection.

Septal Myotomy and Myectomy in Elderly Patients with Hypertrophic Subaortic Stenosis. In the entire operative series of patients with hypertrophic cardiomyopathy the average age at operation is abour 45 years.

With the increasing use of Verapamil, it appears that younger patients are more likely to respond to this drug and have relief of symptoms without the necessity of operation. This is probably one factor operating to increase the average age of patients operated upon recently.

The results of operative treatment in 20 patients with hypertrophic cardiomyopathy 65 years of age or older were evaluated. All 20 patients had marked left ventricular outflow tract obstruction under basal conditions (average gradient 107 mm Hg); the other three patients had outflow gradients of >50 mm Hg only with provocation.

One patient died at operation, and one died four years after operation of circumstances unrelated to hypertrophic cardiomyopathy. Sixteen of the 18 long-term surviving patients have experienced persistent and significant functional improvement for up to six years after operation, including seven patients who are presently asymptomatic. Two patients deteriorated symptomatically after experiencing initial symptomatic improvement for three and four years postoperatively. Operation resulted in a marked decrease or abolition of the left ventricular outflow gradient in each of the 17 patients who underwent postoperative catheterization. These data show that gratifying symptomatic and hemodynamic improvement may be obtained by septal myotomy and myectomy in the elderly patient with hypertrophic cardiomyopathy. Accordingly, advanced age *per se*, is not a contraindication to operative intervention in such patients.

Quantification of Regional Myocardial Dysfunction Produced by Reductions in Coronary Blood Flow: Assessment by Sonomicrometry Techniques. Critical coronary artery obstruction reduces coronary blood flow but the magnitude of coronary blood flow reduction producing myocardial dysfunction is unknown. The quantitative effects of controlled coronary blood flow reductions upon myocardial function using sonomicrometry techniques were examined in seven dogs.

Paired 2 mm piezo-electric ultrasound crystals were implanted 1.5 cm apart within the left ventricle in the distribution of the left anterior descending coronary artery (LAD) along an axis of major fiber shortening. An adjustable occluder encircled the LAD above its first diagonal branch; an electromagnetic flow probe, located distal to this occluder, measured coronary blood flow. The LAD distal to the occluder supplied 36% of the left ventricle. In 42 evaluations (6±2/dog), regional and global left ventricular function was measured before, during, and after reductions of coronary blood flow of 25, 50, 75, 90, and 100%. The results were:

	Control	25%	50%	75%	90%	100%
Diastolic expansion (mm)	1.5±0.5	1.7±0.5	2.0±0.4*	2.0±0.4*	2.2±0.4*	2.1±0.4*
Systolic shortening (mm)	1.4±0.4	1.1±0.4	0.8±0.3*	0.4±0.3**	0.4±0.3**	0.3±0.4**
d length/dt (mm/second)	8.6±2.0	7.3±2.0	5.8±3.0*	3.0±2.0*	2.8±4.0*	2.4±3.0*

*$p < 0.05$ **$p < 0.001$

There were no statistically significant changes in LVEDP, dp/dt, aortic systolic pressure, cardiac output, or endocardial ST segments. These results indicate that significant changes occur in regional ventricular wall motion when coronary blood flow is reduced more than 25%. The ischemic area of myocardium becomes dilated and hypokinetic. Moreover, these changes occur without significant global left ventricular dysfunction or endocardial ST segment elevations. It was concluded that reductions of coronary blood flow by more than 25% produces severe regional myocardial dysfunction which was previously undetectable by global ventricular function measurements. Sonomicrometry represents a most sensitive technique to assess regional myocardial function and ischemia.

LDH Isoenzymes and Evaluation of Perioperative Myocardial Injury. In an attempt to improve the accuracy with which the diagnosis of myocardial infarction is made in patients undergoing cardiac surgical procedures, an improved method for analyzing serum samples for LDH isoenzymes was used to test the accuracy of the reversal of the LD-1/LD-2 ratio in serum as index of myocardial infarction.

The use of the isoenzymes of LDH, particularly the reversal of the ratio of LD-1/LD-2 from less than 1.0 to greater than 1.0 has been shown to be an accurate index of myocardial infarction in the following situations: in the coronary care unit, in patients undergoing general surgical procedures and in patients undergoing myocardial revascularization. Because of the uncertain effect that operative injury to the myocardium might have on serum enzymes, and because it has been held in the past that the hemolysis associated routinely with cardiopulmonary bypass might in and of itself result in reversal of the LD-1/LD-2 ratio, no more widespread use of the test had been attempted.

In a study of 40 patients undergoing various cardiac surgical procedures, including myocardial revascularization, valve replacement, left ventriculomyotomy and myectomy for relief of obstruction due to assymetric septal hypertrophy, and procedures for the correction of palliation of various congenital anomalies, the LD ratio was examined anew as a possible index of myocardial infarction. Electrocardiograms and serum samples were obtained less than 24 hours before operation, 4 hours and 24 hours after completion of cardiopulmonary bypass, and on the mornings of postoperative days 2, 3, 5, 7. An additional serum sample was collected 30 minutes after separation from cardiopulmonary bypass.

Detailed analysis of the postoperative course was carried out on each patient and a judgment made as to whether there was any hemodynamic evidence of infarct. Consideration was given to a number of patient variables as well as to operative findings, operative technique, and postoperative requirements for pharmacologic circulatory support. Each serum sample was analyzed for total Lactate Dehydrodgenase and Creatine Kinase as well as LD isoenzymes and CK-MB. A numerical value for the LD-1/LD-2 ratio in each sample was assigned. The serial electrocardiograms were all reviewed by a single cardiologist who had no knowledge of the patients or their postoperative courses, but was advised of the type of surgical procedure each had undergone.

It was concluded, on the basis of this study, that the LD-1/LD-2 ratio reversal was an accurate index of myocardial infarction in all patients undergoing cardiac surgical procedures except those who had ventriculotomies as a part of a repair of a congenital anomaly. It was determined in that group of patients that a ventriculotomy incision in and of itself did not result in reversal of the ratio. It was further shown that the hemolysis associated with cardiopulmonary bypass did not result in false positive results, that is the reversal of the ratio without the occurrence of infarct.

The results of the study also indicate that all those patients who experienced reversal of the ratio exhibited this finding in that sample collected 24 hours after cardiopulmonary bypass. On the basis of this finding it is concluded that the LD ratio reversal is not only accurate as an index of myocardial infarction but that it can be routinely employed as a rapid and inexpensive screening test in all patients undergoing cardiac surgical procedures.

The adjunctive data produced by the analysis of all samples for total LD, CK and CK-MB indicated that, in contrast to previous reports, CK-MB appeared in the serum of all patients undergoing cardiac surgical procedures. In light of this finding, it was concluded that the appearance of CK-MB is not a reliable indicator of infarct in this patient population. The amount of CK-MB detected in each patient was well above the level at which artifact might be raised as a possible explanation.

Characterization of Naturally Occurring, Genetically Transmitted, Fibrous Subaortic Stenosis in Newfoundland Dogs. The morphologic and the hemodynamic abnormalities occurring in Newfoundland dogs with genetically transmitted subaortic stenosis have been the subjects of several studies. The lesion is manifested by the presence of a precordial murmur, thrill, arrhythmia, congestive heart failure, bacterial endocarditis of the aortic valve, or sudden death. Obstruction to left ventricular outflow is caused by the development of a circumferential, subaortic fibromuscular ring continuous with the anterior leaflet of the mitral valve, virtually identical to the same lesion in humans. The obstruction is associated with left ventricular hypertrophy, intramural coronary artery lesions, myocardial fibrosis, and abnormalities of myocardial blood flow.

Of the 122 Newfoundland dogs studied at the present time, 60 have had hemodynamic and/or morphologic evidence of subaortic stenosis. The percentages of animals with obstruction increased with the ages of the animals. None of 21 newborns had obstruction; one of five less than one month old had obstruction; 15 of 20 aged two to twelve months had obstruction; and 44 of 76 between the ages of one and seven years had obstruction.

The left ventricular outflow tract obstruction varied from a discrete fibromuscular subaortic ring to a tunnel type of left ventricular outflow tract. The left ventricular outflow tract gradients varied from 5 to 95 mm Hg. There was a linear correlation of left ventricular outflow tract gradient with left ventricular mass. All animals with gradients greater than 25 mm Hg had distinctly abnormal left ventricular to body weight ratios.

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In those animals with obstruction, the subaortic ring extended down from the aortic annulus and was continuous with the anterior leaflet of the mitral valve, similar to the lesion of discrete subaortic stenosis in man. Histologically, the subaortic stenosing ring consisted of fibroblasts, myofibroblasts, mature smooth muscle cells, cells resembling chondrocytes, interstitial ground substance, collagen, and small amounts of elastin.

Sixteen animals died suddenly. Four had bacterial endocarditis of the aortic valve. In 12 death was unexplained, and was presumed to be due to arrhythmia. Two of these deaths occurred during exercise. The youngest dog suffering sudden death, without having had endocarditis, was four months old.

Fibromuscular intimal proliferative lesions were present in the intramural coronary arteries of many animals. The extramural coronary arteries were unaffected. The intramural coronary artery lesions were studied in detail in 18 dogs. These coronary artery lesions were associated with left ventricular outflow tract obstruction, increased left ventricular mass, myocardial interstitial fibrosis, and alterations of myocardial blood flow.

Myocardial blood flow was studied using radioactively labeled microspheres in 12 dogs with obstruction and left ventricular hypertrophy and in 12 dogs without. These studies were performed under resting and stressed conditions. The stressed conditions consisted of techycardia, increased afterload, and decreased diastolic pressure. Evaluation, using cluster analysis techniques, of the stressed produced alterations of myocardial blood flow indicated that there was a 170% greater decrease in endocardial to epicardial flow ratios with stress for the dogs with hypertrophy and obstruction as compared to those without obstruction.

Early and Late Hemodynamic Evaluation of Crystalloid and Blood Cardioplegia. Numerous studies have compared the acute hemodynamic effects of different cardioplegic solutions, however. none have evaluated late myocardial function. Fifteen dogs were divided into three equal groups. Group I (control) underwent two hours of 37°C cardiopulmonary bypass. Group II underwent two hours of cardiopulmonary bypass including one hour of continuous 20°C cardiac arrest with crystalloid cardioplegia (CCP). Group III was identical to Group II except that arrest was with blood cardioplegia (BCP). Both cardioplegia solutions were similar in potassium concentration (30 mEq/L), osmolarity (350-365 mosm), and pH (7.50-7.55). Stroke work index (SWI), LVEDP, and Vpm were measured preoperatively, immediately postoperatively, twenty-one days postoperatively, and 120 days postoperatively; compliance curves were performed with an intraventricular balloon at 120 days.

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	Group I (control)	Group II (CCP)	Group III (BCP)
preop SWI (GmM/Kg)	36.6 ± 4.7	46.3 ± 3.2	47.2 ± 3.8
120 day SWI	30.1 ± 2.2	34.6 ± 2.7*	43.5 ± 5.6
preop LVEDP (mmHg)	9.6 ± 0.6	8.6 ± 0.8	9.0 ± 0.8
120 day LVEDP	11.4 ± 0.5	14.2 ± 1.2*	12.2 ± 1.5*
25cc intraventricular balloon LVP (mmHg)	28.6 ± 0.9	40.2 ± 1.2**	32.2 ± 1.5

*$p < 0.05$ compared to preop value **$p < 0.01$ compared to Groups I & II

No significant differences from preoperative values were present for SWI, LVEDP, or Vpm immediately postoperatively or after twenty-one days postoperatively. At 120 days hearts arrested with BCP maintained significantly ($p < 0.01$) better SWI (92.2% of preoperative value) compared to hearts arrested with CCP (74.4%). Additionally, at 120 days left ventricular compliance for the CCP group was significantly ($p < 0.05$) decreased compared to the BCP and control groups. Therefore, while hearts arrested with BCP or CCP had no early deterioration of myocardial function, hearts arrested with CCP demonstrated greater impairment of myocardial function at 120 days than hearts arrested with BCP.

Endothelial Changes in Human Saphenous Veins Prepared for Coronary Artery Bypass Grafts: Effects of Distention Pressure and Preservation Techniques. The saphenous vein has become the conduit of choice in coronary artery revascularization procedures. Despite its popularity and usefulness, clinical and pathological studies indicate that these vein grafts are subject to early closure and mild to severe intimal hyperplasia or atherosclerosis. Experimental models have demonstrated that endothelial damage or disruption can predispose to these conditions. To evaluate the endothelium of saphenous veins prepared in the usual manner at this institute and others, a portion of the harvested vein taken for coronary artery bypass grafts in patients, was immediately fixed in a distended condition at mean arterial pressure for electron microscopy. These veins were compared to veins which were distended and fixed at venous pressure alone.

Scanning electron microscopic comparisons of the effects of handling techniques, immersion media. and distension pressures on human SV morphology were made.

Segments of saphenous veins from 30 patients were divided into 7 groups of 5 veins each. Group 1 was immediately distended and fixed with glutaraldehyde at 100 mm Hg. Groups 2 and 3 were immersed in normal saline for one hour at 4°C and 28°C, respectively, and then distended to 100 mm Hg with saline. Groups 4 and 5 were immersed in blood for one hour at 4°C and 28°C, respectively, followed by distension to 100 mm Hg with blood. Groups 6 and 7 were immediately distended with saline (group 6) or blood (group 7) to 300 mm Hg. One saphenous vein from each group was grasped with a vascular clamp; one saphenous vein in each group also contained a side branch which had been

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ligated. All groups were fixed by perfusion with glutaraldehyde at 100 mm Hg to simulate arterial pressure and examined with a scanning electron microscope.

Saphenous veins immersed in warm saline sustained massive endothelial cell loss, while saphenous veins immersed in warm blood showed only moderate damage. Cold blood and cold saline immersion fully preserved endothelium; however, saline immersion produced mural edema. Distension to 300 mm Hg with saline produced severe endothelial damage and edema, an effect lessened by blood distension. Vascular clamping destroyed endothelium and fractured the intima. Marked luminal stenoses were caused by 4 of 7 side branch ties that appeared normal externally. It is concluded that human saphenous veins are best preserved by a "no-touch" harvesting technique, minimizing manipulation, placement of side branch ties away from the vein wall, immersion in cold blood, and avoidance of distension above 100 mm Hg.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02668-02 SU

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Left Ventricular Myotomy and Myectomy in Patients with Obstructive Cardiomyopathy and Previous Cardiac Arrest

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Andrew G. Morrow, Chief, Clinic of Surgery, NHLBI

OTHER: Jean-Paul Koch, M. D., Clinical Associate, Clinic of Surgery, NHLBI
Barry J. Maron, M.D., Senior Investigator, Cardiology Branch, NHLBI
Kenneth M. Kent, M.D.,PhD. Chief, Cardiovascular Diagnosis Sec. NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MANYEARS: 3-1/2	PROFESSIONAL: 3-1/2	OTHER:

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

From 1960 to May 1979 245 patients have been operated upon at the National Heart, Lung, and Blood Institute for relief of symptomatic idiopathic hypertrophic subaortic stenosis. Left ventricular myotomy and myectomy was carried out in nine patients with obstructive hypertrophic cardiomyopathy solely because of a previously documented episode of cardiac arrest. Prior to arrest, each patient had either no or only minimal functional limitation, and, therefore, would not have met the usual criteria for operation: severe symptoms unresponsive to medical therapy.

DESCRIPTION: Of the nine patients, one died in the perioperative period, and one died suddenly and unexpectedly nine months postoperatively. The remaining seven patients have survived nine months to 5-1/2 years after operation; six of the seven are asymptomatic and one has only mild symptoms. Operation resulted in marked decrease or abolition of the left ventricular outflow gradient under basal conditions in seven of the eight patients studied postoperatively. Significant residual outflow obstruction was demonstrated after operation in the patient who later died.

DISCUSSION: Sudden death in patients with obstructive hypertrophic cardiomyopathy appears usually to result from ventricular arrhythmia, and prevention of recurrent fatal arrhythmia is the goal of treatment in patients who have had cardiac arrest and been resuscitated. Such patients should be treated indefinitely with antiarrhythmic drugs. In addition, when severe outflow obstruction is present, we postulate that effective operative relief of obstruction and consequent lowering of left ventricular systolic pressure will provide additional protection.

PROPOSED COURSE: This work is In Press - American Journal of Cardiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 02669-02 SU

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Results of septal myotomy and myectomy in elderly patients with hypertrophic subaortic stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Jean-Paul Koch, M. D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Barry J. Maron, M.D., Senior Investigator, Cardiology Branch, NHLBI
Stephen E. Epstein, M.D,, Chief, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Surgery Branch, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

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PROFESSIONAL: 3
OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Idiopathic hypertrophic subaortic stenosis is being identified with increasing frequency in older patients. The results of operative treatment in 20 patients with hypertrophic cardiomyopathy 65 years of age or older, are described. All 20 patients were severely symptomatic prior to operation. Seventeen of the 20 patients had marked left ventricular outflow tract obstruction under basal conditions (average gradient 107 mm Hg); the other three patients had outflow gradients of > 50 mm Hg only with provocation.

DESCRIPTION: All patients were severely symptomatic before operation; 17 had marked LV obstruction at rest and in the other 3 large gradients appeared with provocation. All 20 patients had been unresponsive to medical therapy. One patient died at operation, and one died four years after operation of circumstances unrelated to hypertrophic cardiomyopathy. Sixteen of the 18 long-term surviving patients have experienced persistent and significant functional improvement for up to six years after operation, including seven patients who are presently asymptomatic. Two patients deteriorated symptomatically after experiencing initial symptomatic improvement for three and four years postoperatively. Operation resulted in a marked decrease or abolition of the left ventricular outflow gradient in each of the 17 patients who underwent postoperative catheterization.

DISCUSSION: These data show that gratifying symptomatic and hemodynamic improvement may be obtained by septal myotomy and myectomy in the elderly patient with hypertrophic cardiomyopathy. Accordingly, advanced age *per se*, is not a contraindication to operative intervention in such patients.

PROPOSED COURSE: Submitted for presentation at the 53rd Scientific Session of the American Heart Association Meeting.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02671-02 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Long-Term Follow-up and Evaluation of the Hancock (SGP) Bioprosthesis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles L. McIntosh, M.D., Ph.D., Senior Surgeon, Clinic of Surgery, NHLBI

OTHER: Kenneth M. Kent, M.D., Ph.D., Chief, Cardiovascular Diagnosis Section, Cardiology Branch, NHLBI
Stephen E. Epstein, M.D., Chief, Cardiology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
7	7	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Since July of 1970, 553 Hancock "SGP" bioprostheses have been implanted in 436 patients; 105 patients have been followed more than four years. Follow-up data includes early and late mortality and causes, thromboembolic incidence, valve failures and modes of failures, six months postoperative hemodynamic data for atrioventricular and aortic implants, late (> 4.5 years) hemodynamic data for mitral valve patients, and complications following valve replacement with the "SGP" bioprosthesis. Durability of the bioprosthesis implanted more than 4.5 years is marginal. Long-term follow-up reveals a 9% (10/105) valve failure rate in the atrioventricular positions at a mean of 77.8 months following implant. Late hemodynamic prosthetic stenosis has been found in 7 of 18 patients studied an average of 85 months after implantation. Aortic prosthesis has failed early, with 88 valves at risk. All other factors continue to be favorable regarding the Hancock "SGP" prosthesis.

Description: The natural history following replacement of either of the atrioventricular valves or aortic valve is directly related to the inherent complications associated with the prosthetic valve implanted as well as the severity of disease necessitating cardiac valve replacement. The Hancock "SGP" bioprosthesis has been found clinically acceptable because of good hydraulic function in all positions, low incidence of thromboembolism without anticoagulants, nonhemolytic design of the valve, resistance to infection and gradual mode of failure of the "SGP" bioprosthesis. Clinical and late hemodynamic failures of the "SGP" bioprosthesis continue to be our major concern regarding this valve now nearing 10 years of clinical use. Since July of 1970, 553 Hancock "SGP" bioprostheses have been implanted in 436 patients followed for more than four years. Patients return to NHLBI six months postoperatively to undergo examination and cardiac catheterization. Thereafter clinic visits are scheduled each year and periodic hospitalizations for late follow-up are recommended. Major complications are recorded and long-term follow-up data obtained.

Results: Analysis will be confined to patients receiving one or more bioprostheses, and patients with a bioprosthesis and a mechanical prosthesis will be considered when evaluating durability. Patients were implanted with bioprostheses as follows: MVR = 130; AVR = 88; AVR, MVR = 22; MVR, TVR = 44 and AVR, MVR, TVR = 8. Maximum follow-up following MVR is 110.3 months with the median follow-up 27.2 months and 81.0% ± 4.5 patients surviving at 96 months. Six patients have survived eight years and three patients nine years. Myocardial infarction and congestive failure were the most common causes of death following mitral valve replacement with 8 (6.3%) early (130 days) and 14 (11.1%) late deaths. Death secondary to prosthetic valve failure was seen in one patient and prosthetic valve infection and dysfunction in one patient.

Anticoagulants were not routinely used postoperatively and in patients receiving isolated mitral valve replacement 4.4 emboli/100 patient years was observed. Anticoagulation complications are thus avoided which may occur in 5% of patients taking Coumadin per year.

Cardiac catheterization has revealed good early hydraulic function of the Hancock "SGP" bioprostheses in the atrioventricular position. Late hemodynamic data indicates progressive stenosis in 7 of 18 patients studied an average of 85 months after implant. The 18 patients represent one-third of the 54 patients at risk. A patient was deemed to have prosthetic stenosis if their mean MVG increased 5 mm.Hg or their mean MVA decreased by 0.5 cm^2 compared to their 6 months study. Three of the seven patients have been reoperated upon and the remaining four are being followed. The etiology of progressive prosthetic stenosis has been collagen degeneration and calcification in two patients and "stent creep" in one. Eighty-eight patients have had aortic valve replacement with the standard Hancock bioprosthesis at NIH. A peak systolic gradient of 8.0 ± 1 mm. Hg for sizes 21 through 27 has been found in our aortic series. Isoproterenol infusion in 15 patients increased the resting PSG from 10 to 44 mm.Hg. Satisfactory hydraulic function was found in the smaller 21-23 mm. standard bioprostheses contrary to current opinion.

Valve failures have occurred in nine patients (11 valves), eight mitral, two tricuspid and one aortic. One hundred five patients have been followed more than 4.5 years which represents a 9.5% late failure rate. Failures have occurred in the atrioventricular positions 56 to 108 months, an average of 77.8 months after implant. The aortic prosthesis failed 11 months after implant of collagen degeneration. Four of the eight mitral bioprosthetic failures were implanted in 1970. "Stent creep" was responsible for severe prosthetic stenosis in one patient and contributed to failure in two other patients. The thickness and composition of the stent has been changed since 1975 and may prevent failure secondary to stent migration. Collagen degeneration, mineralization and thrombosis continue to be the primary causes of bioprosthetic failures. The mode of failure continues to be gradual with no catastrophic failures to date. Eight patients have been reoperated upon for failure with two deaths, one early and one late. The ninth patient died prior to operative intervention.

Seven patients in our series have had documented sepsis with no prosthetic involvement requiring removal of the prosthesis or recurrence of infection once antibiotics were discontinued. One patient developed three episodes of endocarditis after triple valve replacement with Hancock "SGP" bioprostheses; all three valves were destroyed when examined at necropsy 40 months after implantation. No postoperative myobacterium chelonei infections have occurred in our series. Hemolysis has not been seen following atrioventricular or aortic replacements with the Hancock "SGP" prosthesis.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02681-02 SU

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

LDH isoenzymes and evaluation of myocardial injury in the period immediately following cardiopulmonary bypass (application of a new technique in isoenzyme analysis).

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI
OTHER: Nicholas Papadopoulos, Ph.D., Senior Staff, Clinical Pathology, CC
Byron McLees, M.D., Chief, Critical Care Medicine, CC

COOPERATING UNITS (if any)

Clinical Pathology Department, The Clinical Center

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In an attempt to improve the accuracy with which the diagnosis of myocardial infarction is made in those patients undergoing cardiac surgical procedures an improved method for analyzing serum samples for LDH isoenzymes was used to test the accuracy of the reversal of the LD-1/LD-2 ratio in serum as index of MI to eliminate the problem of spurious results due to hemolysis associated with a cardiopulmonary bypass.

DESCRIPTION: Forty patients undergoing a variety of cardiac surgical procedures were studied through preoperative, intraoperative and serial postoperative serum samples and electrocardiograms. Detailed analyses of the clinical course of each patient was made. Using the same serum samples, values for CK, CK-MB, and total LD were also generated as adjunctive data.

Reversal of the ratio of LD-1/LD-2 in serum samples was found to be a most accurate index of perioperative myocardial infraction in patients undergoing myocardial revascularization, valve replacement, left ventriculomyotomy and myectomy and congenital anomaly repairs when an atriotomy alone was the route of repair. No conclusions could be reached about the accuracy of the reversal of the ratio in those patients undergoing correction of congenital defects through right ventricular incisions, except that right ventriculotomy alone did not result in reversal of the LD-1/LD-2 ratio.

It was also shown that hemolysis due to cardiopulmonary bypass did not result in reversal of the ratio as has been previously reported. Analysis of the same serum samples for CK-MB found the isoenzyme to appear in significant amounts in every patient following cardiopulmonary bypass. This renders CK-MB ineffective as an index of MI in this group of patients. It had been previously thought to be a reasonable indicator of MI in cardiac surgery patients.

The use of the isoenzymes of LDH, particularly the reversal of the ratio of LD-1/LD-2 from less than 1.0 to greater than 1.0 has been shown to be an accurate index of myocardial infarction in the following situations: in the coronary care unit, in patients undergoing general surgical procedures and in patients undergoing myocardial revascularization. Because of the uncertain effect that operative injury to the myocardium might have on serum enzymes, and because it has been held in the past that the hemolysis associated routinely with cardiopulmonary bypass might in and of itself result in reversal of the LD-1/LD-2 ratio, no more widespread use of the test had been attempted.

In this study of 40 patients undergoing various cardiac surgical procedures, including myocardial revascularization, valve replacement, left ventriculomyotomy and myectomy for relief of obstruction due to assymetric septal hypertrophy, and procedures for the correction of palliation of various congenital anomalies, the LD ratio was examined anew as a possible index of myocardial infract. Electrocardiograms and serum samples were obtained less than 24 hours before operation, 4 hours and 24 hours after completion of cardiopulmonary bypass, and on the mornings of postoperative days 2, 3, 5, 7. An additional serum sample was collected 30 minutes after separation from cardiopulmonary bypass.

Detailed analysis of the postoperative course was carried out on each patient and a judgment made as to whether there was any hemodynamic evidence of infarct. Consideration was given to a number of patient variables as well as to operative findings, operative technique and postoperative requirements for pharmacologic circulatory support. Each serum sample was analyzed for total Lactate Dehydrodgenase and Creatine Kinase as well as LD isoenzymes and CK-MB. A numerical value for the LD-1/LD-2 ratio in each sample was assigned. The serial electrocardiograms were all reviewed by a single cardiologist who had no knowledge of the patients or their postoperative courses, but was advised of the type of surgical procedure each had undergone.

RESULTS: It was concluded on the basis of this study that the LD-1/LD-2 ratio reversal was an accurate index of myocardial infarct in all patients undergoing cardiac surgical procedures except those who had ventriculotomies as a part of a repair of a congenital anomaly. It was determined in that group of patients that a ventriculotomy incision in and of itself did not result in reversal of the ratio. It was further shown that the hemolysis associated with cardiopulmonary bypass did not result in false positive results, that is the reversal of the ratio without the occurrence of infarct.

The results of the study also indicate that all those patients who experienced reversal of the ratio exhibited this finding in that sample collected 24 hours after cardiopulmonary bypass. On the basis of this finding it is concluded that the LD ratio reversal is not only accurate as an index of MI but that it can be routinely employed as a rapid and inexpensive screening test in all patients undergoing cardiac surgical procedures.

The adjunctive data produced by the analysis of all samples for total LD, CK and CK-MB indicated that in contrast to previous reports, CK-MB appeared in the serum of all patients undergoing cardiac surgical procedures. In light of this finding, we conclude that the appearance of CK-MB is not a reliable indicator of infarct in this patient population. The amount of CK-MB detected in each patient was well above the level at which artifact might be raised as a possible explanation.

PROPOSED COURSE: The manuscript is being prepared for submission for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02682-02 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Change in canine systemic-pulmonary shunt flow by afterload reduction

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD 20205

TOTAL MANYEARS: 1 and 1/4	PROFESSIONAL: 1	OTHER: 1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A model for the study of the feasibility of nonoperative, pharmacologic manipulation of the flow in a systemic-to-pulmonary shunt is produced by constructing a right subclavian-to-pulmonary shunt in acutely prepared canines. The appropriate measurements of flows and pressures are recorded so that total systemic, and pulmonary resistances might be calculated simultaneously with recorded shunt flows. Such baseline data are recorded after volume infusion has been employed to attain slightly elevated filling pressures in the heart. Afterload reduction is then instituted by sodium nitroprusside infusion and increased incrementally while pressures and flows are constantly recorded, until the increases in afterload reduction are continued until arterial pressures fall below acceptable limits or until significant reduction in shunt flow is noted.

Description: At the present, the management of severe congestive heart failure in patients with existent systemic pulmonary shunts is limited to digitalization, diuresis, and operative intervention to modify the shunt. Most such patients are already digitalized and results of diuresis are usually less than hoped for, especially since such volume loss may result in diminished peripheral perfusion. Afterload reduction has been shown experimentally to cause significant decreases in intracardiac left to right shunts but its potential in reducing flow in a systemic-pulmonary shunt has not been explored.

In the acute setting, foxhounds undergo median sternotomy and creation of systemic (subclavian) to right pulmonary artery shunt with an interposed segment of synthetic graft material for ease of preparation. Flow probes are then placed on the ascending and descending aorta, main pulmonary artery, and on the remaining portion of the subclavian artery proximal to the interposed synthetic graft. Material pressure monitoring is established for the PA, RA, LA, LV, proximal (ascending) aorta and the distal thoracic aorta. Once a steady state has been established, infusion of sodium nitroprusside is begun at 1 mg/kg/min), providing the left atrial mean pressure is 8-10 mm. Hg. Should it be less than that value, volume infusion using whole blood and crystalloid is administeered. The nitroprusside infusion is continued at the initial level for 10 minutes during which continuous recordings are made of the above listed pressures and flows. If no significant reduction in shunt flow or arterial pressure is seen, the infusion rate is increased by 1 mg/kg/min every 10 minutes until either a 25% reduction in shunt flow is noted, arterial mean pressure falls to or below 75% baseline or until an infusion rate of 10 mg/kg/min is reached.

Ten animals have been studied in the manner described above. It was rarely necessary to exceed 4-5 mcg/kg/min to obtain a resultant 20-25% reduction in systemic to pulmonary shunt flow. Initial review of the data indicate that the rise in cardiac output and pulmonary blood flow that are a consequence of the afterload reduction may equal or exceed in absolute numbers the reduction in shunt flow. Should this be verified by final analysis of the data, then any improvement in gas exchange must be attributed to reduction in pulmonary vascular resistance and pulmonary vascular volume due to the known effect of reducing preload associated with nitroprusside.

Course: Complete analysis of data. Should the findings prove statistically significant, it is intended to proceed with preparation of a manuscript written with a main premise that the flow across a systemic-to-pulmonary shunt is not a central problem in cyanosis and failure unless that shunt is of an overly large diameter, in which case reduction of shunt flow may be an added benefit of the use of nitroprusside while the main benefits would still be redistribution of vascular volume and reduction in pulmonary vascular resistance.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02683-02 SU

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Evaluation of polytetrafluorethylene (PTFE) aorto-coronary grafts in dogs

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-1/4	1-1/4	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

No satisfactory synthetic substitute is available for human vein for myocardial revascularization procedures, although there are isolated reports of success using several different synthetic arterial prostheses. A new synthetic material, IMPRA^R, an expanded polytetrafluorethylene compound has been recently modified to enhance antithrombogenicity by impregnating the inner aspect of the graft with particulate graphite. This study is designed to evaluate this new material as a possible substitute for vein in revascularization of myocardium. Ten adult foxhounds underwent left thoracotomy and cardiopulmonary bypass, with subsequent cardiac arrest sustained with cold crystalloid potassium cardioplegia. A short segment of tapered graphite impregnated Impra was imposed as a bypass segment to the LAD coronary artery distal to a ligature placed to occlude that vessel. Six dogs survived and underwent catheterization and coronary angiography between the 30th and 60th postoperative day. All grafts were occluded and in no animal could the native vessel be visualized by retrograde flow.

DESCRIPTION: There is currently no acceptable synthetic substitute for autogenous vein for use in myocardial revascularization, or as a replacement for small arteries in peripheral vascular surgery. There have been isolated reports of successful revascularization of the myocardium using both woven dacron prostheses and Gore-tex^R, an expanded polytetrafluorethylene graft material with an outer cellophane wrap. A relatively new PTFE compound, IMPRA^R has recently been produced with a modification that is intended to enhance the antithrombogenicity of its inner surface. The material is identical to that available for clinical use, except that its inner surface is impregnated with particulate graphite. This study was designed to evaluate this modified graft material as a substitute for autogenous vein in myocardial revascularization. In the initial phase of the study, ten adult foxhounds underwent left thoracotomy and cardiopulmonary bypass with the aid of a roller pump and bubble oxygenator. After aortic cross-clamping, cardiac arrest was sustained with cold potassium rich crystalloid cardioplegia. The left anterior coronary artery was dissected out near its origin and then ligated at its origin. Aorto-coronary continuity was then re-established by constructing a bypass graft from the ascending aorta to the LAD using a 10-12 cm segment of custom designed graphite impregnated IMPRA^R that was extruded so as to taper from 5 mm I.D. at the aortic end to 3 mm I.D. at the coronary end. This was designed to minimize graft to coronary size mismatch.

Eight dogs survived operation. Six survived beyond 48 hours and all of these survived until studied between 30 and 60 days postop. During that interval each survivor underwent cardiac catheterization and coronary angiography. In all six, the graft was found to be occluded, and in no case could the native LAD be seen to fill by collateral blood flow. It was concluded that the arterial runoff bed of the canine LAD may be minimal and review of the operative notes indicate the largest LAD encountered to be of approximately 1.5 mm inside diameter. For these reasons it was decided to repeat the study with the single modification of constructing the distal or coronary anastomosis to circumflex artery rather than the LAD since the circumflex is routinely of twice the diameter.

In the second group of ten foxhounds, nine survived the initial operative procedure. The intraoperative death was due to infarct. Of the remaining nine, two died, both observed sudden deaths during the first 48 hours after operation. Autopsy indicated both to have no firm occlusion of the prosthetic grafts. There was, however, in both cases a pannus of red cells, platelets and fibrin of approximately 1 mm thickness over the entire inner aspect of the graft. This no doubt led to critical reduction in the effective lumen at the graft-coronary anastamosis, with resultant marked decrease in blood flow to the heart. Although no evidence of MI was apparent on sectioning of the myocardium, it seems apparent that that reduction in blood flow led to either infarct complicated by arrhythmia and sudden death or there may have been fatal arrhythmias as a result of ischemia alone. There was one late death at three weeks, due to pneumonia.

Six attempts were made to construct similar bypasses using the autogenous saphenous vein in the dog. There are currently two survivors. The mode of death in three of the four nonsurvivors was observed and was sudden, much like the two described above. There were several differences, however. Those dogs expiring after SVG bypass did so between two and four weeks postop, rather than during the first 48 hours. Further, in the vein graft group, organized thrombus was found at the coronary to graft anastamosis in each case.

There are currently six surviving dogs with synthetic grafts in place and two dogs with vein grafts. They have been awaiting coronary angiography for periods of up to five months. This has not been carried out because contractors have failed to re-establish operative status in the animal laboratory catheterization laboratory. It was to have been operational in early 1980.

PROPOSED COURSE: It has been intended that the animals will be recalled from the Poolesville facility and studied as soon as the needed facilities are operational.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02684-02 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Diastolic retroperfusion of acutely ischemic myocardium utilizing a balloon tipped coronary vein catheter

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Daniel M. Goldfaden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Surgery

SECTION

INSTITUTE AND LOCATION
NHLBI-NIH, Bethesda, MD. 20205

TOTAL MANYEARS: 1-3/4	PROFESSIONAL: 1-3/4	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Despite advances in intraaortic balloon pumping and emergency myocardial revascularization, salvage of ischemic myocardium has been limited in man by the lack of effective collateral circulation into an area of acute infarction. We are currently carrying out experimental trials of perfusing ischemic myocardium by retrograde diastolic pulsation of oxygenated blood into the coronary veins draining an area of ischemia via a balloon tipped catheter that can be introduced transvenously. Results to date indicate correction of EKG changes, reversal of dyskinetic areas and improvement in myocardial performance in acutely ischemic myocardium following institution of retroperfusion.

Description: A double lumen balloon-tipped catheter is placed retrogradely into the coronary sinus in an experimental animal and advanced into the left anterior descending coronary vein to an extent that when the balloon is partially inflated the lumen is obstructed, but unobstructed to normal systolic venous flow when deflated.

This catheter is connected to an oxygenated blood source, the brachial artery, through a synchronized 20 cc. pulsatile assist device which is driven by a standard electrocardiogram-actuated intraaortic balloon counterpulsation pump. After creating an area of ischemic myocardium by LAD ligation, and recording myocardial dynamics with flow transducers and intramyocardial ultrasonography, the coronary vein perfusion pump is actuated. Oxygenated blood is injected retrograde into the coronary vein in diastole via the balloon catheter, whose balloon simultaneously fills and obstructs venous drainage during the injection. The pump is then deactivated during systole. The balloon deflates, and blood drains from the coronary vein into the right atrium in the normal fashion. Myocardial dynamics are reassessed during this procedure.

We designed and experimentally evaluated a transvenously introduced double-lumen balloon tipped catheter and ECG-activated pumping system for perfusing ischemic myocardium by retrograde pulsation of oxygenated blood into the coronary veins during diastole. Balloon deflation during systole allowed normal venous drainage. Sixteen dogs were instrumented with sonomicrometry crystals and catheters to measure regional and global left ventricular (LV) function. The left anterior descending coronary artery (LAD) was occluded for 40 minutes. Following 10 minutes of ischemia, the affected LV regions were dilated and dyskinetic. Coronary vein retroperfusion (CVRP) was instituted for 30 minutes in 8 dogs (controls) were not perfused. CVRP restored 37% of LV systolic shortening (1.5 mm. vs 0.6 mm)*; controls had no systolic shortening. Regional LV dilation of 2.2 mm. was reduced to 1.5 mm. by CVRP; controls expanded to 3.1 mm.* Endocardial ST segments returned to normal with CVRP, but elevated 19 mm. in controls*. Cardiac output rose to baseline levels with CVRP but remained 1.5 L/min depressed in controls*. Three of 8 controls died. Four of 5 remaining dogs died from LV fibrillation after LAD release; all CVRP dogs recovered. CVRP offers a transvenous approach for assisting and perfusing failing, ischemic myocardium. * $(p < 0.001)$

Results of project: Abstract was submitted for presentation at the American Heart Association Scientific Sessions, November, 1980. Acceptance is pending.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 02686-02 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Endothelial changes in human saphenous veins prepared for coronary artery bypass grafts: Effects of distention pressure and preservation techniques.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Victor Ferrans, M.D., PhD., Chief, Ultrastructure Sec., Pathology Branch
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Tokuhiro Ishihara, M.D., Guest Investigator, Ultrastructure Sec.,Path.Br.

COOPERATING UNITS (if any)
Pathology Branch, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 1-3/4	PROFESSIONAL: 1-3/4	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Endothelial changes in saphenous veins harvested for coronary artery bypass grafts were found when veins prepared in this manner were examined using the scanning and transmission electron microscopes. Using current distention pressures and preservation techniques, severe endothelial disruption was found. We compared the endothelial changes caused by a variety of currently employed vein handling techniques to determine the best method to protect venous endothelium during the bypass procedure.

PHS-6040
(Rev. 10-76)

DESCRIPTION: The saphenous vein has become the conduit of choice in coronary artery revascularization procedures. Despite its popularity and usefulness, clinical and pathological studies indicate that these vein grafts are subject to early closure and mild to severe intimal hyperplasia or artherosclerosis. Experimental models have demonstrated that endothelial damage or disruption can predispose to these conditions. To evaluate the endothelium of saphenous veins prepared in the usual manner at this institute and others, a portion of the harvested vein taken for coronary artery bypass grafts in patients was immediately fixed in a distended condition at mean arterial pressure for electron microscopy. These veins were compared to veins which were distended and fixed at venous pressure alone.

Endothelial damage occurring during preparation of saphenous vein grafts is an important cause of early and late graft failure. To determine optimal preparation techniques for human saphenous veins (SV) we made a scanning electron microscopic comparison of the effects of handling techniques, immersion media and distension pressures on human SV morphology. Segments of SV from 30 patients were divided into 7 groups of 5 veins each. Group 1 was immediately distended and fixed with glutaraldehyde at 100 mm Hg. Groups 2 and 3 were immersed in normal saline for one hour at 4°C and 28°C, respectively, and then distended to 100 mm Hg with saline. Groups 4 and 5 were immersed in blood for one hour at 4°C and 28°C, respectively, followed by distension to 100 mm Hg with blood. Groups 6 and 7 were immediately distended with saline (group 6) or blood (group 7) to 300 mm Hg. One SV from each group was grasped with a vascular clamp; one SV in each group also contained a side branch which had been ligated. All groups were fixed by perfusion with glutaraldehyde at 100 mm Hg to simulate arterial pressure and examined with a scanning electron microscope. SV immersed in warm saline sustained massive endothelial cell loss, while SV immersed in warm blood showed only moderate damage. Cold blood and cold saline immersion fully preserved endothelium; however, saline immersion produced mural edema. Distension to 300 mm Hg with saline produced severe endothelial damage and edema, an effect lessened by blood distension. Vascular clamping destroyed endothelium and fractured the intima. Marked luminal stenoses were caused by 4 of 7 side branch ties that appeared normal externally. We conclude that human SV are best preserved by a "no-touch" harvesting technique, minimizing manipulation, placement of side branch ties away from the SV wall, immersion in cold blood, and avoidance of distension above 100 mm Hg.

RESULTS: The project has culminated in the presentation of two papers on the preservation of human saphenous veins and their publication.

Gundry, S.R., Ishihara, T., Jones, M. and Ferrans, V.: Intraoperative trauma to human saphenous veins: Comparison by scanning electron microscopy. ANN. Thorac. Surg. 30:40-47, 1980.

Gundry, S. R., Jones, M., Ishihara, T., and Ferrans, V. J.: Optimal preparation of human saphenous vein grafts. Presented at Society for Vascular Surgery, Chicago, Ill. June 26, 1980. In Press. Surgery.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02687-02 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Unidirectional intra-atrial flap valve: Evaluation in experimental pulmonary hypertension and right ventricular failure

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Steven R. Gundry, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Daniel M. Goldfaden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 1-1/4	PROFESSIONAL: 1-1/4	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES x☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Surgical repair of intracardiac defects with pulmonary hypertension is still associated with a 20 to 30 percent mortality, often secondary to right ventricular dilatation and failure. Unloading of the right ventricle may be accomplished by venous unloading only at the expense of left ventricular output. Therefore, we are evaluating a unidirectional intra-atrial flap valve that allows right-to-left shunts when right ventricular filling pressures are elevated, thus decompressing the right ventricle while maintaining or improving left ventricular output.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Experimental animals are given a pressure load to the right ventricle by means of an adjustable band around the pulmonary artery and physiologic parameters are measured over a variety of resistances to right ventricular outflow. Using extracorporeal circulation, a unidirectional flap valve of dacron reinforced Lycra is implanted in the atrial septum which allows a right-to-left shunt only when right atrial pressures exceed left atrial pressures. Under normal conditions, the valve remains closed, but during increasing right ventricular dilatation and obstruction, right atrial pressure exceeds left atrial pressure and shunts blood right-to-left, thus acting as a "pop-off valve." In initial design studies, right-to-left shunting occurred when right ventricular pressures in dogs exceeded 50 mm. Hg, and arterial hypoxemia was within tolerable limits. Cardiac output in these dogs was maintained and right ventricular dilatation did not result. Similar pressures in control animals caused right ventricular dilatation and numerous arrhythmias.

Right ventricular (RV) failure occurs following repair of congenital heart defects with associated pulmonary or RV hypertension. To alleviate this failure, we experimentally evaluated the effects of a prosthetic patent foramen ovale implanted in the atrial septum of 21 dogs. The valve had a 6 mm orifice and a flap on the left atrial side which allowed only a right-to left shunt. In 8 dogs studied acutely, the valve contained a balloon mechanism to occlude its lumen intermittently. In 40 trials in these dogs, production of RV hypertension by pulmonary artery banding with the valve occluded reduced aortic (105 to 52 mm Hg) and left atrial (8.4 to 4.4 mm Hg) pressures and increased RV end-diastolic (6.5 to 10.2 mm Hg) and right atrial (7.6 to 14 mm Hg) pressures ($p < 0.001$). Opening the foramen ovale valve returned all pressures to control ($p < 0.005$) while maintaining peak RV pressures. Right-to-left shunt fractions were 0.25 ± 0.13. All eight dogs studied acutely died when their valves were permanently occluded. There were no deaths amont 13 dogs with initially patent valves followed by catheterizations up to 4 months later. Valves closed after 2.4 ± 1 weeks. Valve closure after 5 days (11 dogs) was not associated with RV failure. A prosthetic patent foramen ovale will prevent RV decompensation in acute and subacute RV hypertension.

PROPOSED COURSE: The past year and one-half has been spent in design and fabrication of an anti-thrombogenic valve which can be implantable but which can become tissue covered with time. After encouraging initial testing, we tested the valve in acute studies to determine its potential for development. This was followed by chronic implantation experiments. The results of these experiments have been submitted to the American Heart Association for presentation at the annual meeting in November 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02696-01 SU

PERIOD COVERED October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Morphometric analyses of myocardial hypertrophy (compensated and decompensated) utilizing computer-assisted microdensitometric techniques.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D. Senior Surgeon & Investigator, Clinic of Surgery,
Joseph B. Zwischenberger, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: James M. DeLeo, Computer Systems Analyst, CSL, DCRT
Victor J. Ferrans, M.D., PhD., Chief Ultrastructural Sec, Pathology Br.
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI

COOPERATING UNITS (if any)

Division of Computer Research Technology & Pathology Branch

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung & Blood Institute

TOTAL MANYEARS: 2	PROFESSIONAL: 1-1/2	OTHER: 1/2

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☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been developing computer-assisted interactive techniques using microdensitometry and surface display facilities to perform micromorphometric analyses of hypertrophied myocardium from humans and from experimental animal preparations. Our techniques have the ability to analyze approximately 202,500 samples (pixels) from a photomicrograph of a tissue specimen. The size of the sample is determined by the magnification of the photomicrograph. Depending upon the information required, magnifications vary from 10 to 860 X, so that resolutions may be varied from 5 to 0.5 millimicrons. Techniques developed thus far allow the ability to determine percent fibrosis, percent interstitium, cardiac muscle cells diameter and shape, uniformity of cell shape and size, and ratios of cardiac muscle area to numbers of nuclei and to numbers of capillaries.

DESCRIPTION: Myocardial tissues which we have obtained for investigation include the following: 1) human cardiac muscle from patients operated upon because of right ventricular outflow tract obstruction, 2) human cardiac muscle obtained at operation for obstructive hypertrophic cardiomyopathy, 3) left ventricular myocardium from Newfoundland dogs with naturally occuring, genetically transmitted, subaortic stenosis, 4) hypertrophied right ventricular myocardium from swine having had pulmonary artery constrictions, and 5) left and right ventricular myocardium for which the stimulus for hypertrophy has been produced _in utero_ in fetal lambs.

The computer programs and packages have been developed for the micromorphometric determinations of cardiac muscle cell diameters and shape, uniformity of cell shape and size, percentage of interstitium, and ratios of cardiac muscle cell area to numbers of nuclei and to numbers of capillaries. This portion of our investigations has been initiated upon animal tissues. The computer programs for percentage fibrosis in the hypertrophied myocardium near completion at the present time and pilot studies have been initiated.

PROPOSED COURSE: All of the above described tissues will be analyzed by the developed computer-assisted, interactive, micromorphometric techniques. We anticipate extending these techniques to three dimensional image analysis of normal and abnormal cardiac muscle cells. This investigation will involve reconstruction of cardiac muscle cells from thin (one millimicrometer) thick serial sections of human and animal cardiac muscle cells. We also plan to evaluate isolated cardiac muscle cells by similar computer-assisted techniques.

The data obtained from the micromorphometric analyses will be integrated with studies of clinical evaluations and with studies of ventricular function. Thus we will continue to investigate the interrelationships of myocardial morphology with compensated and decompensated myocardial hypertrophy.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02697-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Characterization of naturally occuring, genetically transmitted, fibrous subaortic stenosis in Newfoundland dogs.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery NHLBI

OTHER: A. Michael Borkon, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Victor J. Ferrans, M.D., PhD., Chief Ultrastructure Sec. Pathology Br.
William C. Roberts, M.D., Chief, Pathology Branch, NHLBI
Joseph E. Pierce, DVM, Chief, Sec. Laboratory Animal Medicine & Surgery, NHLBI

COOPERATING UNITS (if any)
Pathology Branch and Section of Lab Animal Medicine & Surgery, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2	1-1/2	1/2

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have been characterizing the morphologic and the hemodynamic abnormalities occurring in Newfoundland dogs with genetically transmitted subaortic stenosis. Of 122 animals studied thus far almost one-half have hemodynamic and/or morphologic evidence for left ventricular outflow tract obstruction. The obstruction is absent at birth, appearing after one month of age. The lesion presents clinically by the presence of a precordial murmur, thrill, arrhythmia, congestive heart failure, bacterial endocarditis of the aortic valve, and sudden death. Obstruction to left ventricular outflow is caused by the development of a circumferential, subaortic fibromuscular ring continuous with the anterior leaflet of the mitral valve, virtually identical to the same lesion in humans. The obstruction is associated with left ventricular hypertrophy, intramural coronary artery lesions, myocardial fibrosis, and abnormalities of myocardial blood flow.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Of the 122 Newfoundland dogs studied at the present time, 60 have had hemodynamic and/or morphologic evidence of subaortic stenosis. The percentages of animals with obstruction increased with the ages of the animals. None of 21 newborns had obstruction; one of five less than one month old had obstruction; 15 of 20 aged two to twelve months had obstruction; and 44 of 76 between the ages of one and seven years had obstruction.

The left ventricular outflow tract obstruction varied from a discrete fibromuscular subaortic ring to a tunnel type of left ventricular outflow tract. The left ventricular outflow tract gradients varied from 5 to 95 mm Hg. There was a linear correlation of left ventricular outflow tract gradient with left ventricular mass. All animals with gradients greater than 25 mm Hg had distinctly abnormal left ventricular to body weight ratios.

In those animals with obstruction, the subaortic ring extended down from the aortic annulus and was continuous with the anterior leaflet of the mitral valve, similar to the lesion of discrete subaortic stenosis in humans. Histologically, the subaortic stenosing ring consisted of fibroblasts, myofibroblasts, mature smooth muscle cells, cells resembling chondrocytes, interstitial ground substance, collagen, and small amounts of elastin.

Sixteen animals suffered sudden death. Four were due to bacterial endocarditis of the aortic valve. Twelve, unexplained, are presumed to be due to arrhythmias. Two of these deaths occurred during exercise. The youngest dog suffering sudden death, without having had endocarditis, was four months old.

Fibromuscular intimal proliferative lesions were present in the intramural coronary arteries of many animals. The extramural coronary arteries were unaffected. The intramural coronary artery lesions were studied in detail in 18 dogs. These coronary artery lesions were associated with left ventricular outflow tract obstruction, increased left ventricular mass, myocardial interstitial fibrosis, and alterations of myocardial blood flow.

Myocardial blood flow was studied using radioactively labelled microspheres in 12 dogs with obstruction and left ventricular hypertrophy and in 12 dogs without. These studies were performed under resting and stressed conditions. The stressed conditions consisted of tachycardia, increased afterload, and decreased diastolic pressure. Evaluation, using cluster analysis techniques, of the stressed produced alterations of myocardial blood flow indicated that there was a 170% greater decrease in endocardial to epicardial flow ratios with stress for the dogs with hypertrophy and obstruction as compared to those without obstruction.

PROPOSED COURSE: A manuscript is in preparation about the regional myocardial blood flow in left ventricular hypertrophy. Another manuscript is in preparation regarding the interrelationships of abnormalities of myocardial blood flow with intramural coronary artery lesions, myocardial hypertrophy, and interstitial fibrosis. Further studies are planned using computer-assisted micromorphometric techniques to quantitate the aspects of hypertrophy and of interstitial fibrosis in these animals. Studies are also underway to investigate mechanisms of the early development of the obstructing subaortic ring.

PUBLICATIONS:

Borkon, A.M., Bell, J. H., Pierce, J. E., Jones, M. and Morrow, A.G.: Regional myocardial blood flow in left ventricular hypertrophy. Circulation 59, 60 (Suppl II): 1012, 1979.

Jones, M., Borkon, A. M., Ferrans, V. J., Pierce, J. E., and Roberts, W.C.: Fibrous subaortic stenosis in Newfoundland dogs: Characterization of a congenital heart disease acquired after birth. Presentations World Congress of Paediatric Cardiology, (Strong S, Ed.) London, 1980, Grange Press, Sussex, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02698-01 SU

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Postoperative cardiac care in infants and children

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D., Senior Surgeon & Investigator, Clinic of Surgery, NHLBI
Lily Ng, R.N., M.S.N., Supervisor Clinical Nurse, Clinical Center

OTHER: Jaroslav Stark, M.D., Consultant Cardiothoracic Surgeon, Thoracic Unit, The Hospital for Sick Children, London, England
Adelaide Tunstill, Sister in Charge, Thoracic Unit, Hospital for Sick Children, London, England

COOPERATING UNITS (if any)

Nursing Department, CC, Thoracic Unit, Hospital for Sick Children, Longon, England

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung & Blood Institute

TOTAL MANYEARS: 1/4	PROFESSIONAL: 1/4	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Cardiac surgery in infants and children has progressed so that most lesions may be corrected early in life. In theory and in practice the postoperative management of infants and children is similar to that of adult patients. Nevertheless, the potential for growth, the particular and often immature metabolic processes, and the cardiovascular physiology of congenital malformations demand special postoperative considerations. From our experiences in the Hospital for Sick Children, London, England and in the Clinic of Surgery of the NHLBI, we have developed guidelines for postoperative management to assist nurses and clinical associates with care of young patients after cardiac operations.

PHS-6040
(Rev. 10-76)

DESCRIPTION: The special problems of postoperative cardiac care in infants and children have been divided into the following categories: 1) preoperative considerations; 2) choice of operative procedure; 3) initial postoperative assessment; 4) basic procedures; 5) cardiovascular problems, including heart rate, preload, afterload, contractility, subacute and chronic heart failure, and rhythm disturbances; 6) respiratory problems, including care of infants and children on ventilators, weaning from ventilatory support, and problems of phrenic nerve paralysis; 7) cardiopulmonary resuscitation; 8) fluid, electrolyte, and other metabolic problems; 9) nutrition; 10) body temperature regulation; 11) renal problems, including peritoneal dialysis; 12) hematological problems; 13) jaundice; 14) infection; 15) neurological problems; 16) medication dosages; 17) prognoses without and following operation; and, 18) parental counseling.

From the above delineated considerations we have developed postoperative management guidelines, particularly directed toward the initiate and limitedly experienced nurse and clinical associate assisting with the postoperative care of pediatric cardiac surgical patients.

PROPOSED COURSE: The postoperative management protocols for our unit are in the process of being refined. A publication specifically concentrating upon nursing considerations is in preparation.

Jones, M., Stark J.: Special problems of postoperative care in infancy and childhood. In: Postoperative Cardiac Care. (Brainbridge MV, Ed), C. V. Mosby, St. Louis (IN PRESS)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02699-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Evaluation of Operative Treatment for Discrete Subaortic Stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glenn R. Barnhart, M.D., Clinical Associate, Clinic of Surgery, NHLIB

OTHER: Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3/4	3/4	

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We evaluated the late clinical status of 34 patients undergoing operation for discrete subaortic stenosis. Followup ranged from 1 to 245 years with a mean of 13.7 years; 3 of the 34 patients could not be located at the time of this evaluation. The average age at the time of operation was 16.8 years of age (range 5 to 55). There were 4 operative deaths (10.5%) during our early experience. The average preoperative peak systolic gradient was 98.5 mm Hg. Postoperative catheterization was performed in all patients from 2 to 168 months following operation. Postoperative gradients ranged from 0 to 200 mm Hg (mean 35 mm Hg). Seven patients had postoperative gradients greater than 50 mm Hg, ranging from 58 to 200 (mean 103 mm Hg); 5 of these 7 patients had the tunnel variety of subaortic stenosis at the time of operation. Five of the 31 patients available for late clinical evaluation died (13.8%); four of these deaths were sudden. Nineteen patients had aortic regurgitation preoperatively (61.3%); aortic regurgitation was present in 21 patients (67.7%) examined within 6 months after operation. Actuarial survival at 10 years is 87%, and at 24 years is 81%.

We conclude that the resection of the subaortic membrane in this disease effectively removes left ventricular outflow tract obstruction. However, because of the striking association of aortic regurgitation and this disease, patients should continue to be followed on a long-term basis.

DESCRIPTION: A chart review was performed to collect preoperative, operative, and postoperative data base on 38 patients having undergone operation for discrete subaortic stenosis. Patients have been and are currently being seen in the Cardiac Surgery Clinic to assess late clinical status of all patients. Chest x-rays, electrocardiograms, and echocardiograms are being performed on all patients to more objectively evaluate late results.

RESULTS: Examination of the data collected on these patients emphasizes several important features of this disease. Most patients who undergo operation for subaortic stenosis and who are found to have the typical crescent shaped membrane can be expected to do well following operation. There should be no significant residual gradient. However, if at the time of operation patients are found to have the tunnel variety of subaortic stenosis, the chance of abolishing their gradient is much less. In our study almost all patients having significant gradients postoperatively had tunnel subaortic stenosis.

A second salient feature of this disease is the strong association with aortic regurgitation. Resection of the membrane did not improve the competence of the aortic valve. There was only one patient in whom aortic regurgitation disappeared in the postoperative period. The presence of aortic regurgitation in discrete subaortic stenosis has been well recognized previously; the damage to the aortic valve appears to occur because of the constant jet effect of blood from the subaortic membrane. It is interesting to note that only one patient in our series has required aortic valve replacement for aortic regurgitation.

In conclusion, discrete subaortic stenosis is a type of left ventricular outflow tract obstruction amenable to operative relief. Patients found to have a discrete subaortic membrane at the time of operation should be expected to have no obstruction following resection and a good long-term result. Because of the association of aortic regurgitation with this disease even in the postoperative period, all patients should be followed on a regular basis with serial assessment of degree of aortic valve incompetence, deterioration of functional status, and ventricular function.

PROPOSED COURSE: Following accumulation and examination of data presently being collected to evaluate the late clinical status of these patients, this paper will be submitted for presentation to the American College of Cardiology, San Francisco, California, March 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02700-01 SU

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Tricuspid valve replacement in the young animal; hemodynamic and ultrastructural changes of bioprosthetic valves following implantation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Glenn R. Barnhart, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Daniel Rose, M.D., Research Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Victor J. Ferrans, M.D., Chief, Ultrastructural Section, Pathology Branch, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Pathology Branch, NHLBI

LAB/BRANCH

Surgery

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1	1/2

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Valvular heart disease, either of congenital or acquired origin, may be sufficiently severe to require surgical intervention. With an increase in understanding of valvular disease, there has been a decrease in morbidity and mortality for patients requiring valve replacement. However, as long-term survival has increased, problems related to artificial valve thromboembolism, anticoagulation, valve induced hemolysis, and, more recently, fibrocalcific changes in bioprosthetic valves have become apparent.

Description: Recent electron microscopic studies of implanted valves have shown changes in leaflet structure, (1, 2). Duration of implantation seems to correlate with severity of alteration in ultrastructure.

Because of the present popularity of bioprosthetic valves and the increasing reports of valve failure due to fibrocalcific changes, we initiated this study to better define the process leading to calcification,

Twenty young male rams have undergone tricuspid valve replacement in the NHLBI animal research laboratory. In ten animals we implanted #21 Ionescu-Shiley Bovine Pericardial Xenograft Bioprostheses, and in the remainder we implanted #21 Hancock Porcine Bioprostheses. Each animal underwent right heart catheterization to assess the valve gradient immediately following valve implantation.

Proposed Course: Animals within each valve population will be divided into three groups and ergo catheterization at 2, 4, and 6 months. Following catheterization, each valve will be removed to undergo histopathologic and ultrastructural examination. Each valve will be analyzed both qualitatively and quantitatively for calcium deposition. With this study the sequence of events in bioprosthetic valve alterations which ultimately leads to chemical dysfunction will be more readily defined.

---

1. Ferrans, V. J., Spray, T. L., Billingham, M.E., and Roberts, W. C.: Structural changes in glutaraldehyde-treated porcine heterografts used as substitute cardiac valves. Am. J. Cardiol. 41: 1159-1184, 1978.

2, Ferrans, V. J., Boyce, S. W., Billingham, M.E., Jones, M., Ishihara, T., and Roberts, W. C.: Calcific deposits in porcine bioprostheses: Structure and pathogenesis. Publication pending.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02701-01 SU

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Design and testing of a model to show that the expired CO_2 from each lung accurately reflects the fraction of pulmonary blood flow to each lung.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Richard E. Michalik, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Y. Kim, M.D., Deptartment of Anesthesiology, Georgetown University and Clinical Center.

COOPERATING UNITS (if any)

Anesthesiology Department, Clinical Center

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

TOTAL MANYEARS: 3/4	PROFESSIONAL: 3/4	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this study was to test the hypothesis that expired carbon dioxide from each lung accurately reflects the blood flow to that lung. To do this, an experimental model was created whereby pulmonary blood flow could be determined directly using electromagnetic blood flow determinations for the right and left pulmonary arteries individually. Simultaneous determinations of the expired CO_2 from each lung were made using a double lumen endotracheal tube which permitted separate but simultaneous ventilation of each lung. The expired gases were analyzed continuously from a sampling port in each lumen to a Chemetron Mass Spec II spectrophotometer.

PHS-6040
(Rev. 10-76)

DESCRIPTION: It has been felt for some time that the expired CO_2 content in the gaseous mixture expired from each lung is a reflection of the blood flow to that lung. This hypothesis has been tested by several indirect methods, including measurement of expired carbon dioxide and simultaneous measurement of expired labeled Xenon following injection of the Xenon into a femoral vein.

In order to further examine this hypothesis, a model was designed to permit direct and continuous mesurement of the blood flow to the right and left lungs in such a manner that these flows could be instantaneously compared with continuous on-line determinations of the expired CO_2 from each lung.

An adult male foxhound was anesthetized using pentobarbital. After EKG monitoring and arterial pressure monitoring were established, the animal underwent median sternotomy with preservation of the mediastinal ligaments so as to provide structural support to maintain the heart in position and prevent torsion about the great vessels. The pericardium was opened ventrally to allow for additional cradle like support. The right and left pulmonary arteries were then dissected out and carefully stripped of excess adventitia. Electromagnetic perivascular flow probes were placed about the two pulmonary arteries. Pulmonary artery pressure monitoring was established with a flow directioned double lumen catheter introduced through the right atrial appendage, and left atrial pressure was monitored with an indwelling catheter.

The previously placed endotracheal tube was removed as a tracheostomy was completed, and the double lumened tube was introduced into the trachea and passed distally until its curved tip came to rest in the ostia of the left mainstem bronchus. In this position the tube allowed for individually controlled but simultaneous ventilation of the right and left lungs. Needle type sampling ports were introduced into the two lumens which has been connected to individually Harvard animal ventilators. Each ventilator had individual supplies of gases so that the left lung could be ventilated only with room air, oxygen or mixture of the two. Nitrogen was included in the gas mixture to the ventilator supplying the right lung. In this way Nitrogen could be mixed in any amount with, or substituted for oxygen or room air so as to render the right lung hypoxic.

The percent expired CO_2 was determined for each lung in the baseline state, and the ratio of the two CO_2 contents was determined. Pulmonary blood flows from the right and left lung were simultaneously recorded and their ratio determined. Initial analysis of the data indicated these two ratios to be quite alike.

Several manipulations were then undertaken to determine whether or not changes in pulmonary blood flow, that is a change in the ratio of the right and left pulmonary artery blood flows would be accurately reflected by the change in the ratio of the expired CO_2 values in the right and left lungs. Agains, the data indicate that as one ratio changed, so changed to other, indicating the expired Co_2 was an accurate reflection of the pulmonary flows. As a further test, the right lung was made hypoxic bvy substituting nitrogen for oxygen as the inspired gas. In this case, there was a reduction in blood flow to the right, or hypoxic lung, and the resultant change in the ratio of right and left pulmonary blood flows was mirrored by the change in the ratio of right and left lung expired CO_2.

We feel that this method of simultaneous on-line direct measurements of expired CO_2 and pulmonary blood flow from two separately ventilated lungs shows that the expired CO_2 from each lung is an accurate reflection of blood flow to that lung. this permits a variety of harmacologic agents and ventilatory manipulations to be examined as to their effects on pulmonaryu blood flow simply by knowing the total cardiac output and the ratio of expired CO_2 of the right and left lungs. Any new ratio that results from manipulation of a variable need only be coupled with another cardiac output determination to arrive at the blood flow to eachg lung at that moment.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02702-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Identification of Cardiac Myosin Isoenzymes in Man

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John J. Schier, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Robert S. Adelstein, M.D., Head, Sec. of Molecular Cardiology, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

Cardiology Branch, NHLBI

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart Lung, & Blood Institute

TOTAL MANYEARS: 1/2	PROFESSIONAL: 1/2	OTHER:

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A previous article from this laboratory (Maron & Adelstein, 1976) reported no significant differences in the cardiac myosins of patients with obstructive hypertrophic cardiomyopathy and normals based on purified myocin ATP'ase activity and light chain composition by one-dimensional SDS-polyacrylamide gel electrophoresis. Since that report, several animal models of cardiac hypertrophy have shown the presence of myosin isoenzymes, due to variants in heavy chain structure. Hoh and associates, using a technique of pyrophosphate gel electrophoresis, demonstrated myosin isoenzymes in the rat which change in distribution with age and with the impaired contractile state of hypothyroidism. Flink and Morkin, in the rabbit, used 2-dimensional mapping of cyanogen bromide digests of myosin to demonstrate a new myosin isoenzyme in hypertrophied rabbits. Schwartz and her associates demonstrated a similar isoenzyme shift in the rat with hypertrophy induced by aortic banding.

DESCRIPTION: The question of the molecular basis for the hypercontractile state in human hypertrophic cardiomyopathy has been reopened. Portions of the surgical myectomy specimens from patients with obstructive cardiomyopathy, and autopsy samples of control hearts from patients with no primary cardiac pathology are being obtained. The techniques of myosin pyrophosphate gel electrophoresis, myosin isoelectric focusing in urea gels, and 2-dimensional peptide mapping are being adapted for use in analyzing purified myosin from these human sources to identify the presence of myosin isoenzyme variants in man.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02703-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Late results following operation for aortic regurgitation associated with ventricular septal defect

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: John J. Schier, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Thomas J. VonRueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M. D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Cardiology Branch, NHLBI, Nuclear Medicine, Clinical Center, NIH

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS: 1/2	PROFESSIONAL: 1/2	OTHER:

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are assessing the late clinical status of 21 patients with aortic regurgitation (AR) and ventricular septal defects (VSD) undergoing operative correction of both lesions in the period between 1959 and 1979. Aortic valvuloplasty was performed in 10 patients (mean age 11 years), and aortic valve replacement (AVR) in 11 (mean age 25 years). Five patients had previous bacterial endocarditis involving the aortic valve, all requiring AVR. The angiographic grade of AR was mild to moderate in the valvuloplasty group, and moderate to severe in the replacement group. The VSD was suture closed in 18, and patched in 3 patients.

PHS-6040
(Rev. 10-76)

DESCRIPTION: There were 3 operative deaths, 2 in children requiring valve replacement with small prostheses. There was one late death in each group, one 6 years postop with prosthetic valve thrombosis, and one 3 months postop with refractory congestive failure and mitral regurgitation. Postoperative catheterization has been performed in 16 patients. There are no residual left-to-right shunts. Six patients in the valve repair group have moderate or greater residual AR, and 4 in the valve replacement group have insignificant AR. None in the valvuloplasty group have required a second operation for residual AR, however.

Outpatient follow up of both groups of patients is continuing. Noninvasive procedures (echocardiography and radionuclide angiography, as indicated) will be used to assess and follow left ventricular function, particularly in those valvuloplasty patients with significant AR, in whom noninvasive indices may have prognostic value in the timing of eventual valve replacement.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02704-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Operation for Congenital Valvular Aortic Stenosis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., Chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1	1/4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☒ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We evaluated the late clinical status of 47 young patients who had aortic commissurotomy for congenital valvular stenosis. These patients have been followed from 5 - 21 years, mean follow-up 13 years. Aortic commissurotomy relieves obstruction and abolishes symptoms in most patients. The operation must be considered palliative because after 10 years, significant valve related problems begin to occur.

950

DESCRIPTION: We evaluated the late clinical status of 47 young patients who had aortic commissurotomy for congenital valvular aortic stenosis. Duration of follow-up is at least 5 years and ranges up to 21 years (mean 13 years).

The average age at the time of operation was 14 years. There were no operative deaths. Forty-five patients had bicuspid valves and 2 patients had unicuspid valves. Cardiac catheterization was performed on one or more occasions in 38 patients 6 to 192 months (mean 45 months) after operation. The postoperative average peak systolic valve gradient was 34 mm Hg (range 0 to 110 mm Hg); the average aortic valve index (17 patients) was 0.65 cm^2/M^2 (range 0.23 to 1.03 cm^2/M^2). The average left ventricular end-diastolic pressure was 14 mm Hg (range 3 to 36 mm Hg).

Four cardiac deaths (9%) occurred 5 to 17 years after operation. Seven patients (15%) have required aortic valve replacement after 5 to 17 (mean 11) years. Three of the 47 patients (6%) have required treatment for bacterial endocarditis. Two patients died from noncardiac causes. Currently 34 of the 47 patients are alive without second operations. Symptomatically, they all are in NYHA functional class I. Murmurs of aortic regurgitation are present in 18 patients, each of whom has x-ray evidence of left ventricular enlargement.

Thus, aortic valve commissurotomy successfully relieves obstruction and abolishes symptoms in most young patients with congenital aortic stenosis. The procedure should be considered a palliative one, however, since valve replacement will probably be required in all patients eventually.

COURSE: This abstract has been presented at The First World Congress of Pediatric Cardiology, London, June 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02705-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Left Ventricular Hypertrophy Produced in Utero

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Joseph B. Zwischenberger, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Delwin K. Buckhold, D.V.M., Lab. Animal Medicine & Surgery, NHLBI

COOPERATING UNITS (if any)
Section of Lab Animal Medicine & Surgery

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/2	1 1/4	1/4

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Most experimental investigations of left ventricular hypertrophy use animal models in which obstruction to left ventricular outflow is created after birth. While these models do produce left ventricular hypertrophy, they do not reflect the dynamic, compensatory changes that occur prior to birth. We have developed a model of creating left ventricular hypertrophy in utero. The majority of animals (58%) came to term and can be used for chronic investigations of ventricular hypertrophy.

DESCRIPTION: We have developed an experimental model of ventricular hypertrophy which represents these adaptations. Lamb fetuses from 52 ewes at half gestation (70 days) had 5.5 mm Teflon bands placed around the thoracic aorta; they were allowed to be born spontaneously at term (138 days). Twin pregnancies occurred in 21 of the ewes; when a twin was present only one fetus was banded, the other serving as a control. Thirty banded fetuses and 16 normal twins came to term.

Morphometric analysis of these lambs at a mean age of 20 weeks and a mean body weight of 3.6 Kg indicated that significant left ventricular hypertrophy was present. LV weight for banded animals (B) was 12.8 ± 1.9 Gm and 7.3 ± 1.0 in control animals (C) ($p < .05$). LV free wall thickness for B was 8.1 ± .4 mm compared to 6.3 ± .2 mm for C ($p < .01$). Liver/body weight ratios and lung/body weight ratios were not different in the two groups of animals, suggesting that myocardial decompensation had not occurred in the animals with left ventricular hypertrophy. LV systolic pressures of B were 147% greater than C (126 ± 11 mm Hg vs. 51 ± 11 mm Hg); pulmonary wedge pressures averaged 9 mm Hg for $\overline{B}$ and 8 mm Hg for $\overline{C}$ (p = NS). Cardiac outputs were not different. The gradients produced ranged from 15 to 85 mm Hg (mean 42 ± 9 mm Hg).

An experimental stimulus for left ventricular hypertrophy may be accomplished *in utero*. These animals may be allowed to come to term and be used for acute and chronic investigations of left ventricular hypertrophy, analogous to the left ventricular hypertrophy of congenital heart disease.

COURSE: This abstract will be submitted to The American College of Cardiology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 02706-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Early and Late Hemodynamic Evaluation of Crystalloid and Blood Cardioplegia

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel M. Rose, M.D., Clinic of Surgery, NHLBI.
OTHER: Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Glenn R. Barnhart, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION
National Heart, Lung, and Blood Institute

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER:

CHECK APPROPRIATE BOX(ES)
☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

In order to compare both the acute and chronic effects of different cardioplegic solutions, we studied foxhounds preoperatively, postoperatively, and at 21 and 120 days or immediately postoperatively, hearts arrested with blood cardioplegia had less impairment of ventricular function and compliance at 120 days.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Numerous studies have compared the acute hemodynamic effects of different cardioplegic solutions, however, none have evaluated late myocardial function. Fifteen dogs were divided into three equal groups. Group I (control) underwent two hours of 37°C cardiopulmonary bypass. Group II underwent two hours of cardiopulmonary bypass including one hour of continuous 20°C cardiac arrest with crystalloid cardioplegia (CCP). Group III was identical to Group II except that arrest was with blood cardioplegia (BCP). Both cardioplegia solutions were similar in potassioum concentration (30 mEq/L), osmolarity (350-365 mosm), and pH (7.50-7.55). Stroke work index (SWI), LVEDP, and Vpm were measured preoperatively, immediately postoperatively, twenty-one days postoperatively, and 120 days postoperatively; compliance curves were performed with an intraventricular balloon at 120 days.

	Group I (control)	Group II (CCP)	Group III (BCP)
preop SWI (GmM/kg)	36.6 ± 4.7	46.3 ± 3.2	47.2 ± 3.8
120 day SWI	30.1 ± 2.2	34.6 ± 2.7*	43.5 ± 5.6
preop LVEDP (mmHg)	9.6 ± 0.6	8.6 ± 0.8	9.0 ± 0.8
120 day LVEDP	11.4 ± 0.5	14.2 ± 1.2*	12.2 ± 1.5*
25cc intraventricular balloon LVP (mmHg)	28.6 ± 0.9	40.2 ± 1.2**	32.2 ± 1.5

*$p < 0.05$ compared to preop value   ** $p < 0.01$ compared to Groups I & II

No significant differences from preoperative values were present for SWI, LVEDP, or Vpm immediately postoperatively or after twenty-one days postoperatively. At 120 days hearts arrested with BCP maintained significantly ($p < 0.01$) better SWI (92.2% of preoperative value) compared to hearts arrested with CCP (74.4%). Additionally, at 120 days left ventricular compliance for the CCP group was significantly ($p < 0.05$) decreased compared to the BCP and control groups. Therefore, while hearts arrested with BCP or CCP had no early deterioration of myocardial function, hearts arrested with CCP demonstrated greater impairment of myocardial function at 120 days than hearts arrested with BCP.

COURSE: To be presented at the Surgical Forum, October 22, 1980, in Atlanta, Georgia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02707-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Reperfusion with Blood Cardioplegia Provides Improved Recovery of Both Early and Late Ventricular Function after Ischemic Injury.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel M. Rose, M.D., Guest Worker, Clinic of Surgery, NHLBI
OTHERS: Glenn R. Barnhart, M.,D.,Clinical Associate,Clinical of Surgery,NHLBI
Jean-Paul Koch, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

SECTION

INSTITUTE AND LOCATION
National Heart, Lung, and Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1 1/4	1	1/4

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It has been proposed that modification of the initial reperfusion solution after aortic occlusion, may alter the extent of ischemic injury. We, therefore, studied initial reperfusion with either cold blood or cold blood cardioplegia after ischemic injury. Reperfusion with cold blood cardioplegia provides better recovery of ventricular function and compliance immediately, 21, and 120 days postoperatively than cold blood alone.

PHS-6040
(Rev. 10-76)

DESCRIPTION AND RESULTS: We studied initial reperfusion after aortic occlusion in 15 dogs divided into three equal groups. Group I underwent 2 hours of normothermic cardiopulmonary bypass (CPB); Group II underwent 2 hours of CPB including one hour of 20°C ischemic arrest; Group III was identical to Group II but initially was reperfused with 250cc of 25°C blood cardioplegia (BCP, K^+ = 30 mEq/L) prior to aortic unclamping. Stroke work index (SWI), LVEDP, and Vpm were compared preoperatively, immediately postoperatively, at 21 days and 120 days postopatatively. Results of SWI (GmN) are summarized below ± SEM:

	preop	postop	21d	120d
Gp I	36.6 ± 4.7	30.6 ± 4.2	34.8 ± 4.2	30.1 ± 2.2
Gp II	41.9 ± 3.8	16.4 ± 3.3*	28.9 ± 4.1*	27.9 ± 3.7*
Gp III	37.0 ± 2.9	22.9 ± 3.5*	31.5 ± 2.5	30.1 ± 2.3

Both Gps II & III had significant* elevations in all postoperative values for LVEDP while there were no significant* differences in the values for Vpm between Gps II & III. Hearts which were initially reperfused with BCP (GpIII) had significantly* better recovery of SWI immediately postoperatively (62%vs39%), at 21d (85%vs69%), and at 120d (81%v66%) than Gp II. We conclude that after ischemic injury, initial reperfusion with BCP provides better recovery of both early and late ventricular function than reperfusion with oxygenated blood alone.

*$p < 0.05$

COURSE: Work has been submitted for presentation at the 1980 scientific session of the American Heart Association.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02708-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Late Results of Mitral Valve Repair in Incomplete Persistent Atrioventricular Canal

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel M. Goldfaden, M.D., Clinical Associate, Clinical of Surgery, NHLBI

OTHERS: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Andrew G. Morrow, M.D., chief, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

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Clinic of Surgery

SECTION

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National Heart, Lung, and Blood Institute

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Late clinical evaluation was performed on 34 patients who had repair of uncomplicated incomplete persistent atrioventricular canal at the NIH prior to 1975. This was 87% of hospital survivors evaluated at a mean of 12 years after operation.

There were 3 late deaths and 2 who required late reoperation for mitral valve regurgitation. Using life table statistical analysis the predicted survival free of reoperation at 24 years is 84%.

Of the 30 living survivors evaluated 24 are asymptomatic and the other 6 have mild cardiac symptoms; 7 have significant arrhythmias including 3 who have required pacemakers. None have shown progressive cardiomegaly on chest x-ray but on M-mode echocardiograms in 15 the left atrial size was abnormally enlarged. Persistent systolic heart murmurs were present in 25 but since the majority were asymptomatic and had no other objective evidence of significant mitral regurgitation these murmurs were not felt to be indicative of mitral valve incompetence in most.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Charts of all 44 patients who had repair of uncomplicated incomplete persistent atrioventricular canal defects at the NIH between 1956 and 1975 were reviewed. There were 39 hospital survivors. Early postoperative cardiac catheterization was performed at an average of 11 months in 35. Normal left atrial or pulmonary artery wedge pressures in 29 indicated satisfactory mitral valve function; 6 had minimally elevated wedge pressures while 2 had markedly elevated pressures suggesting significant residual mitral valve incompetence.

Of all survivors 4 had been followed until death or reoperation. Of the other 35 survivors late followup of at least 4 years was available either by chart review or by recent clinic visit. In all of these 30 patients clinical evaluation included chest x-ray, electrocardiogram, history and physical examination. M-mode echocardiograms were performed in the 15 who have had recent clinic visits.

COURSE: Results of this project were recently presented at the World Congress of Pediatric Cardiology. Written report will soon be submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02709-01 SU

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Alterations of Pulmonary Arterioles Produced by Prenatal Coarctation of the Aorta

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI
Renu Virmani, M.D., Assistant Pathologist, Cardiovascular Department, Armed Forces Institute of Pathology, Walter Reed Army Medical Center

OTHERS: Thomas J. Von Rueden, M.D., Clinical Associate, Clinic of Surgery, NHLBI;
Joseph B. Zwischenberger, M.D., Clinical Associate, Clinic of Surgery, NHLBI
Delvin K. Buckhold, D.V.M., Staff Veterinarian, Section of Laboratory Animal Medicine and Surgery, NHLBI.

COOPERATING UNITS (if any)

Cardiovascular Department, Armed Forces Institute of Pathology, Walter Reed Army Medical Center
Section of Laboratory Animal Medicine and Surgery, NHLBI.

LAB/BRANCH

Clinic of Surgery

SECTION

INSTITUTE AND LOCATION

National Heart, Lung, and Blood Institute

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1	3/4	1/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Alterations of pulmonary arterioles have been thought to cause right ventricular hypertrophy in infants with coarctation of the aorta. To investigate the pulmonary vasculature in coarctation of the aorta, we created postductal coarctation of the aorta in utero in 23 fetal lambs at 10 to 11 weeks of gestation. Comparing the experimental lambs at term (21 weeks) to 13 months postnatally with 36 age matched normal lambs, we found that pulmonary artery pressures and right ventricular weights were elevated in those lambs with coarctation. Similarly, pulmonary arteriolar medial thicknesses were statistically significantly increased in lambs with coarctation as compared to normal lambs.

PHS-6040
(Rev. 10-76)

DESCRIPTION: To investigate the changes in the pulmonary arterioles in coarctation of the aorta, we created postductal coarctation in 23 fetal lambs at 10 to 11 weeks of gestation (term gestation is 21 weeks). These experimental lambs were compared to 36 normal lambs (usually twins of experimental lambs), which were matched for age with the lambs with coarctation at the time of study. Obvservations were made at catheterization and at necropsy immediately after birth and up to 3 months of age. Pulmonary artery pressures measured in 11 animals with coarctation averaged 22/9 mm Hg and in 10 normals averaged 19/7 mm Hg. The cardiac outputs for all lambs with coarctation were 130 ± 20 ml/min/kg and for all normal lambs were 100 ± 10 ml/min/kg. The gradients across the coarctation averaged 43.1 ± 6.1 mm Hg.

Morphometric analyses indicated that the lambs with coarctation had hypertrophied right ventricles. The ductus arteriosus was not patent in any animal. The lungs of seven normal and eight lambs with coarctation were fixed with 10% formaldehyde at a pressure of 40 cm of water (29 mm Hg) and randomly sectioned. The external diameters and the medial thicknesses of the muscular pulmonary arterioles were determined for a total of 560 vessels. For vessels less than 250 millimicrons in diameter the medial thickness to external diameter ratios were calculated. These ratios were significantly greater ($p<0.01$ by the Mann-Whitney test) in lambs with coarctation (0.19 to 0.36 - mean 0.28) compared to normal lambs (0.10 to 0.26 - mean 0.21). Thus, in coarctation of the aorta the pulmonary arterioles appear to be altered during the fetal period and remain abnormal after birth. This abnormality of the pulmonary arterioles may be a pathogenic mechanism of right ventricular hypertrophy in infants with coarctation of the aorta.

COURSE: An abstract presenting these data has been submitted for the 53rd Scientific Sessions of the American Heart Association.

Further analyses of the development of the pulmonary arteriolar bed are being performed in lambs with coarctation at varying ages. An analysis of the pulmonary arteriolar vascular bed is in progress in lambs having had in utero production of right ventricular outflow obstruction by constriction of the pulmonary artery.

Extended micromorphometric analyses of the development of the pulmonary vascular bed in normal and abnormal cardiovascular conditions is planned using computer-assisted techniques in collaboration with Mr. James M. De Leo, Computer Systems Analyst, CSL, DCRT.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02710-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Ventricular function and coronary blood flow in chronic ventricular hypertrophy in minature swine

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Daniel M. Goldfaden, M.D., Clinical Associate, Clinic of Surgery, NHLBI

OTHER: Michael Jones, Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Clinic of Surgery

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Pulmonary artery or infra-coronary aortic bands were placed in young minature swine to induce right or left ventricular hypertrophy. As the animals mature and develop ventricular hypertrophy and/or heart failure they are being studied for changes in gross and microscopic ventricular morphology as well as changes in ventricular function and regional coronary blood flow. Swine were chosen for the experimental animals because: 1) at maturity the animals weigh 50 to 60 Kg, about the same as an adult human. 2) The lack of epicardial collateral coronary arteries in swine makes their coronary circulation similar to that of man.

PHS-6040
(Rev. 10-76)

DESCRIPTION: All 24 banded swine have had late hemodynamic and coronary blood flow studies. There were 13 with right ventricular hypertrophy and 9 with right ventricular hypertrophy and failure. Only 2 of the several attempted infra-coronary aortic banded swine developed left ventricular hypertrophy. The technique of infra-coronary banding was found to be unsatisfactory because the small diameter bands used to fit below the coronary arteries eroded into the aorta with growth in most animals, thus failing to produce an aortic obstruction. Five control swine were also studied.

Specimens of tissue from right and left ventricles and ventricular septum have been preserved for evaluation of changes in the cellular structure as seen by light and electron microscopy. Estimation of degree of fibrosis will be performed by a new computer assisted scanning technique.

PROPOSED COURSE: Data from the animals studied is being analyzed to evaluate changes in regional coronary blood flow and ventricular hemodynamics as ventricular hypertrophy and failure develop in response to a pressure overload. When the microscopic studies are completed the changes in ultrastructure will be correlated with the observed changes in ventricular function and coronary blood flow. Upon completion of these evaluations this work will be submitted for publication.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02711-01 SU

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Quantification of regional myocardial dysfunction produced by reductions in coronary blood flow: Assessment by sonomicrometry techniques

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Steven R. Gundry, M.D., Clinic of Surgery, NHLBI

OTHER: Harry W. Seipp, Chief Technician, Clinic of Surgery, NHLBI
Rodger E. Solomon, Electrical Engineer, Biomedical Engineering, DRS
Michael Jones, M.D., Senior Surgeon, Clinic of Surgery, NHLBI

COOPERATING UNITS (if any)
Biomedical Engineering & Instrumentation Branch, DRS

LAB/BRANCH
Clinic of Surgery

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INSTITUTE AND LOCATION
National Heart, Lung & Blood Institute

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1-3/4	1	3/4

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Critical coronary artery obstruction reduces coronary blood flow, but the magnitude of coronary blood flow reduction producing myocardial dysfunction is unknown. We examined the quantitative effects of controlled coronary blood flow reductions upon myocardial function using sonomicrometry techniques. The ischemic area of myocardium becomes dilated and hypokinetic when coronary blood flow is reduced more than 25%. Sonomicrometry techniques detect changes in myocardial function with enhanced sensitivity over techniques which measure global myocardial function.

PHS-6040
(Rev. 10-76)

DESCRIPTION: Paired three mm piezo-electric ultrasound crystals were implanted 1.5 cm apart in the left ventricle in the distribution of the left anterior descending coronary artery along the septal-free wall axis of fiber shortening in 7 dogs. An adjustable occluder encircled the left anterior coronary artery above its first diagonal branch; an electromagnetic flow probe, located distal to this occluder measured coronary blood flow. The left anterior descending coronary artery distal to the occluder supplied 36% of the left ventricle. In evaluations on each dog, regional and global left ventricular function was measured before, during, and after reductions in coronary blood flow of 25, 50, 75, 90 and 100%.

RESULTS:

	Control	25%	50%	75%	90%	100%
Diastolic expansion (mm)	1.5±0.5	1.7±0.5	2.0±0.4*	2.0±0.4*	2.2±0.4*	2.1±0.4*
Systolic shortening (mm)	1.4±0.4	1.1±0.4	0.8±0.3*	0.4±0.3**	0.4±0.3**	0.3±0.4**
d length/dt (mm/second)	8.6±2.0	7.3±2.0	5.8±3.0*	3.0±2.0*	2.8±4.0*	2.4±3.0*

* $p < 0.05$ ** $p < 0.001$

There were no statistically significant changes in LVEDP, dp/dt, aortic systolic pressure, cardiac output, or endocardial ST segments. These results indicate that significant changes occur in regional ventricular wall motion when CBF is reduced more than 25%. The ischemic area of myocardium becomes dilated and hypokinetic. Moveover, these changes occur without significant global left ventricular dysfunction or endocardial ST segment elevations. We conclude that reductions of CBF by more than 25% produces severe regional myocardial dysfunction which was previously undetectable by global ventricular function measurements. Sonomicrometry represents a most sensitive technique to assess regional myocardial function and ischemia.

PROPOSED COURSE: Results of this investigation will be presented at the American College of Surgeons Surgical Forum, October 1980, Atlanta, Georgia and will be published in Surgical Forum 1980.

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1979 TO SEPTEMBER 30, 1980

In a program applying a basic science concept of instrumentation this laboratory recognizes advances in basic physical science and then evaluates their potential to serve biomedical science. Instrumentation for measurement, separation or control of biosystems of interest to biomedical research form the basis for interlaboratory cooperation. Instrumentation concepts are selected for their promise of contribution to advancement of bioscience in general or in response to anticipated requirements. Industrial instrumentation on the other hand tends to develop only ideas with potential sales appeal.

In the section on Biophysical Instrumentation, work is continuing on the development of instrumentation for one of the major unsolved problems of biological interest involving hemeproteins which is, the mechanism of the ligand or electron transport reaction at the active site of the protein. The question is whether this is purely a chemical reaction and if so, what is its nature or is it, in the case of hemoglobin and myoglobin, simply, an entrapment and isomerzation much as in the cryptands? To assist the molecular biologist in answering these questions, Dr. Balko has been developing a low temperature Mossbauer classical transmission apparatus and a totally new selective excitation double Mossbauer (SEDM) instrument. In the classical method the probe of the energy levels in the nucleus by the gamma ray is done by adding or subtracting a small amount of energy to the probing gamma rays by moving the source, i.e. doppler shift in the energy. While this is a necessary first approach, it does not delineate the underlying mechanisms of energy exchange in the nucleus which are so profoundly affected by the environment of the outer shell electrons. Using SEDM and directly probing a particular Mossbauer line and observing the energy exchange to the other Mossbauer line in the nucleus this mechanism can be elucidated. This means that, in the case of hemoglobin, each intermediate can be observed and the contribution of each to the Gibbs Free energy which involves the iron - porphyrin can be ascertained. Since one will also be able to obtain the equilibrium constant of each intermediate by this means, the contributions due to protein structure can also be determined. In the case of oxidative phosphorylation where both non heme iron and the cytochromes are involved the energy movement (electrons or protons) down the electrochemical gradient to oxygen can be followed. In short the instrument promises us a new molecular probe so sensitive to the physical and chemical environment of the iron that it can detect minute differences in the microviscosity of solutions or the electronic energy of the iron. In order to define clearly, both mathematically and experimentally, the new techniques a series of compounds have been studied. These include ferrochrome A, hemin, and hemoglobin. Work has been conducted at temperatures as low as 200 millidegrees Kelvin and at magnetic field intensities as high as 10 Tesla with results not predicted by classical Mossbauer theory, but readily understood with Dr. Balko's new theoretical treatment. A clear demonstration of the enormous effect produced by changes in the microviscosity was demonstrated with sickle cell hemoglobin and is presently being used to study the initation process in sickling.

A new picosecond spectrometer has been built by Dr. Smith and Dr. Liesegang in an attempt to study the detailed movement of the energy and structure of the protein in the reaction of hemoglobin with carbon monoxide or oxygen. This will be correlated with the new laser flash photolysis apparatus, both in solution and in the red cell using a specially constructed dual wavelength spectrometer. These latter studies will be themselves correlated with classical high speed stopped flow studies being conducted on our greatly improved instrument which operates with a dead time of 200 microseconds.

Using our new fast stopped-flow calorimeter, developed by Drs. Balko and Berger, Dr. Liesegang has worked on the thermodynamics and kinetics of the reaction of cryptand 211 with Ca^{++} and Li^{+}. He has shown that a fast bimolecular step occurs and then an isomerzation occurs that has a high heat of reaction for Li^{+} but a very low one for Ca^{++}. This promises to be an important model for the reactions with proteins where isomerzation occurs.

In thermodynamic studies of proteins it is often important to know the heats of reaction and the buffering power or pK's of the reactants. Berger has developed a differential thermal and pH titration apparatus for this with a 3 picomole sensitivity in a 1 ml volume. A new differential calorimeter permits heats of reaction of only 100 μl of each reagent to be quickly determined with a sensitivity of 10 picomoles for a reaction heat of 5 kilocalories per mole.

The fluorescence properties of human serum albumin from different commercial sources were found to vary markedly. This could explain many of the anomalous results in the literature. A charcoal treatment procedure developed here was shown to reduce the differences in spectra, quantum yield, and fluorescence decay kinetics.

The properties of phospholipases were studied with the dye-liposome method. Enzymic hydrolysis of liposomes containing concentration-quenched dye is accompanied by appearance of strong fluorescence. This assay is probably the most convenient one available for this class of enzymes and was used to show activation and inhibition of different phospholipases by serum albumin.

The widely used o-phthaldialdehyde reaction for fluorescence assay of primary amines was examined by stopped-flow fluorometry. This kinetic analysis showed that the second order rate constants for reaction of the

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reagent with various amino acids depended on steric and charge factors as well as pH. The information should be useful for determining optimal conditions of reactions, and for future studies on the rate of reaction of o-phthaldialdehyde with proteins.

With Dr. Oliver Alabaster, we showed that measurement of electronic cell volume in the Los Alamos fluorescence flow cytometer can be accompanied by fading due to electrolysis of the solutions.

Separation techniques based on complex behavior of two phase liquid extraction phases in coiled tubes exposed to centrifugal forces continues to provide the basis for instrumental methods with special advantages.

Countercurrent chromatography and cell elutriation methods have been further developed by constructing new continuous flow coil planet centrifuges, without rotary seals. The toroidal coil planet centrifuge is a table top model of a versatile countercurrent chromatograph which holds a long coiled column around a drum shaped holder. It enables universal application of two-phase solvent systems to separate a variety of biological samples in a small scale. The capability of the aparatus has been demonstrated on separation of DNP amino acids and oligopeptides. A large preparative scale countercurrent chromatographic scheme was successfully made by the use of large-bore glass coils rotating in the gravitational fields. The capability of the scheme has demonstrated on separation of 600 mg of DNP amino acids. Also, the horizontal flow-through coil planet centrifuge previously reported (ZOL-HL-01428-02) has been successfully applied to purification of two synthetic peptides which had poor solubility in the solvents commonly used in the conventional liquid chromatography. For cell separation, a non-cynchronous flow-through coil planet centrifuge was newly designed and its capability was demonstrated on separation of human and sheep erythrocytes with physiological buffered saline. This method has been successfully applied to separations of fluorescence labelled B and T lymphocytes, malaria gametocytes, and rat liver cells.

The Section on Pulmonary and Cardiac Assist Devices continues to explore applications of the Kolobow spiral coil blood gas exchanger and the techniques of gentle blood handling developed in this laboratory.

It has become evident that all the metabolic CO_2 produced can be removed by a low blood flow extracorporeal system and hence this technique can be used to control mechanical pulmonary ventilation so as to avoid barotrauma. The question of pulmonary ventilation on the evolution of hyaline membrane disease was investiaged by using the principles of apneic oxygenation in premature lambs. The development of hyaline disease follows a few hours of pulmonary ventilation so we reasoned that a period of apneic oxygenation at the critical period might tide the newborn over the period when immature lung incapable of coping

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with respiratory ventilation could mature enough to withstand the mechanical functions required.

Investigations have been made to use these ideas to prevent HMD in the preterm fetal lamb model. The fetal lamb at 130 days will develop HMD with a near 100% mortality with state of the art treatment. Nineteen fetal lambs were treated with a gestational age of 131-138 days, and the lungs were kept expanded, while removing all metabolic CO_2 through the placental circulation. Within 4-8 hours the total lung compliance rose to levels found in newborns at term and pulmonary ventilation then became possible without develping HMD. Recent experiments on 8 lambs indicates that on acceleration of the maturation or conditioning can be shortened to as little as 1-1/2 hours by increasing the initial lung expansion pressure. It is of interest to note that the surface tension of the lung washings remained abnormal but hyaline membrane disease did not develop.

In collaboration with the University of Milan a similar technique has been applied to 3 patients with Adult Respiratory Distress Syndrome. These patients met criteria defining 90% mortality. These patients were placed on extracorporeal bypass with removal of metabolically produced CO_2, while providing oxygen through the lungs kept inflated to a constant pressure and ventilating the lungs at 2-4 BPM (LFPPV-Low frequency Positive Pressure Ventilation). Pulmonary oxygen transport improved rapidly, associated with clearing of lungs, and a rise in total lung compliance. This improvement in arterial blood oxygenation was such that all those patients met established criteria for disconnection from bypass by the first day of this treatment.

Acute pulmonary failure in adults as well as in the newborn is a combination of unfortunate events which makes lung involvement extensive in scope and yet fully recoverable with a treatment that allows lungs to mature/heal over a period of time. Managing lungs so involved by the technique described can lead to rapid improvement in lung function and tide the patient over the critical few hours or days. This treatment is not unlike current treatment for chronic renal failure using the artificial kidney, except that it represents a single shot application of treatment of a few hours or days duration after which recovery is full and and complete, without any need for additional treatment. The patients have remained cured and well.

Work on methods and apparatus for the study of kidney and toad bladder epithelial cells grown as sheets on porous membranes continues to yield important results. The central element is the porous bottomed culture device (PBCD) in which the membrane is cemented to polycarbonate rings which provides electrical and chemical isolation between the solutions contacting each side of the membrane. With these PBCD's new knowledge of the development of epithelia is being obtained. For example, the

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development of vasopressin response in the cells (from African clawed toad kidney) requires that there be free exchange for materials between the cells and the media on the basal side (i.e. the PBCD's must spaced off the botton of the dish). By this means, vasopressin response has been demonstrated for the first time in these cells.

Work on PBCD's with transparent collagen membranes has been successful and is continuing. Medulary thick ascending limb cells from the rabbit kidney tubule have grown out from a short piece of tuble on 1 mm collagen membranes in about 10 days and produced apical side positive potentials as high as 23 millivolts.

Analysis of picomole amounts of biologically important materials has been greatly simplified by the development of a new flow-through colorimeter and a fluorimeter. These devices have cuvets with working volumes less than 250 nl and light path lengths of 1 cm. Using continuous-flow techniques, the nanoliter sized samples are injected directly into a flowing stream of reagent which produces a colored product whose optical absorbance is measured. We have demonstrated subpicomole sensitivity for magnesium, calcium, and phospate ions. The new instrument is considerably simpler than existing ultramicroanalytic techniques.

The second activity involves the modeling and testing of a new type of oxygen electrode construction. The new design consists of a cathode intimately contacting a protective barrier membrane. The cathode, a porous silver mesh supports the membrane. Oxygen diffusing through the membrane reacts at the cathode when appropriate electrical circuit is established. The new design permits a response time of less than 10 seconds without significant response shift due to sample stirring. Computer simulation of the electrode-membrane combination permits exploration of the materials and dimensions to obtain the optimum design.

Tissue blood flow instrumentation by analysis of the spectral changes in laser light scattered by the motion of the red cells has again been refined and evaluated in clinical applications. The prototype instrument has been greatly improved by a new analysis scheme, a four meter flexible fiber optic probe, and the use of a photodiode detection system. The linearity of the instrument in respone to blood flow has been greatly improved and noise has been reduced so the instrument can resolve the instantaneous pulsatile flow of microcirculation. The instrument is being used to monitor average blood flow and dynamic blood flow in normal individuals and clinical center patients. Data is being gathered to assess the instruments usefulness as a measure of blood flow in peripheral vascular disease, carotid artery occlusion diesase, and sickle cell anemia, and muscular dystrophy.

We have demonstrated that nuclear magnetic resonance from flowing blood can be used to measure flow in brain entirely by projected electromagnetic fields. The system is entirely harmless and involves no ionizing radiation.

This accomplishment by our contract facility at the Medical College of Wisconsin used projected fields and field ranging techniques to limit the sensitive region to sense only the modulation of the NMR signal by the pulsatile flow of the middle cerebral artery. The large superconducting magnet is now functional and has made these measurements possible. The same system applied to peripheral vessels as well as heart and aorta provide large clear signals which may be of interest to other cardiovascular studies.

The development of magnetic marker and localizing tagging fields is still being studied here by developing signal enchancement systems based on coupling resonating nuclear and electron resonance fields or to nuclear fields of materials with short relaxation times.

At the fields and frequencies available here the results to date are encouraging but inadequate. The superconducting facility may be needed to develop the method fully.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01404-12 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Membrane lung systems for long term respiratory support

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	T. Kolobow	Chief, Pulmonary and Cardiac Assist Devices	LTD	NHLBI
	M. Solca	Visiting Fellow	LTD	NHLBI
Others:	A. Pesenti	Instituto Anestesia E Rianimazione, Milan		
	L. Gattinoni	Instituto Anestesia E Rianimazione, Milan		
	R.E. Ulane		NPMB	CH
	G. Brumley	Duke University		

COOPERATING UNITS (if any)
Duke University, Department of Perinatology, Durham, North Carolina

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Pulmonary and Cardiac Assist Devices

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 2	PROFESSIONAL: 2	OTHER:

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Section on Pulmonary and Cardiac Assist Devices is exploring alternative methods in the prevention and treatment of hyaline membrane disease (HMD) and in the prevention and treatment of adult respiratory distress syndrome (ARDS). Both approaches are based on the concept that pulmonary ventilation can be markedly reduced and even totally eliminated if metabolically produced CO_2 is removed by an extracorporeal membrane lung (CDML) and while oxygen is delivered through the technique known as "apneic oxygenation". In an animal model at high risk of developing HMD, this approach prevented HMD. In ARDS, this approach resulted in rapid improvement in lung function, and total recovery from ARDS.

PHS-6040
(Rev. 10-76)

Objective:

1. To develop a membrane lung system for long term support.

The membrane lung (ML) is a safer device for both short term and long term use than more traditionally used bubble oxygenators. However, the superiority of the ML in short term use can easily be masked during procedures which result in a high cardiotomy blood return. In long term use for respiratory assist, the advantage of the ML system is universally acknowledged, and remains the only safe system for prolonged use.

2. To develop an artificial lung system optimized for long term carbon dioxide removal.

There is no difficulty in removing metabolically produced CO_2 by either the ML or bubble oxygenators during routine bypass. At times, some CO_2 is added to the ventilating gas to prevent too large a drop in PCO_2. This laboratory has pioneered in the control of spontaneous breathing, and in the control of mechanical pulmonary ventilation (MV) through the means of removing part, or all, of metabolically produced CO_2 by the extracorporeal carbon dioxide membrane lung (CDML). All the CO_2 produced at rest in an adult can be removed at a relatively low extracorporeal blood flow of less than 1 liter of blood flow/min. It thus becomes possible to control spontaneous breathing and mechanical pulmonary ventilation when some or all of metabolically produced CO_2 is removed by CDML. This aspect is important in MV because the settings on the MV are determined so as to effect "adequate alveolar ventilation" - meaning sufficient removal of metabolically produced CO_2 to maintain arterial blood PCO_2 in the normal range.

It is furthermore well established that MV has a deleterious effect on organ function remote from the lungs, such as that of the liver, and of the kidneys. Management of patients with adult respiratory distress syndrome (ARDS) is frequently affected at some stage by multiple organ system failure in addition to primary pulmonary failure. We hoped that through extracorporeal CO_2 removal ($ECCO_2R$) it may be possible to eliminate dependence on MV and to reduce multiorgan system failure, and hence, lead to the road of recovery from ARDS.

Newborns with acute pulmonary insufficiency (hyaline membrane disease - HMD) have a different underlying pulmonary pathology. Extracorporeal removal of CO_2 can easily be accomplished in HMD, and afford a means to establish normal pulmonary function.

Similarly, in chronic respiratory failure, extracorporeal removal of CO_2 can restore arterial blood PCO_2 to normal and can provide the means for sustained improvement in alveolar ventilation.

3. Acute respiratory failure: adult respiratory distress syndrome, and hyaline membrane disease.

The outlook for patients with ARDS is poor. In a recent review of all patients requiring MV at an FIO_2 equal to or more than 0.5 for at least 24 hours, two thirds of those patients were dead within one month. This result highlights the difficulty when treating patients whose primary underlying disease involves severe lung failure. It is intriguing to note that those few fortunate survivors after some time of further recovery have no evidence of significant functional impairment attributable to their earlier lung disease. As far as is known, ARDS has no unique underlying bacterial or viral etiology. Rather, it is a combination of unfortunate events that make their lung involvement extensive in scope, but not unique in pathology. To compound the problem, the deleterious side effects of MV result in impaired renal and hepatic function, and a high incidence of barotrauma, and is not in itself conductive to the healing of the lungs.

In the widely quoted controlled ECMO (extracorporeal ML oxygenation) study involving patients with ARDS there was no beneficial effect attibuted to extracorporeal blood oxygenation; however, the control and the treated patients were simultaneously also receiving conventional pulmonary ventilation with a mechanical ventilator. This study merely showed that the ML use could not alter the course of their disease whenever MV was used.

We believe lack of recovery from severe ARDS is due to continuing use of MV. We feel these patients can fully recover by the application of an extracorporeal ML in the control of breathing, in the obtaining of adequate alveolar ventilation without undue mechanical pulmonary ventilation, and in the enhanced pulmonary oxygen transport which follows when patients are placed on apneic oxygenation, or on low frequency positive pressure ventilation (LFPPV). By controlling the amount of CO_2 removed by an extracorporeal CDML, spontaneous breathing, or MV can be adjusted to allow for diseased lungs to heal.

In the management of acute respiratory failure in the newborn (hyaline membrane disease = HMD), substantial progress has been made in the last decade through the application of continuous positive airway pressure (CPAP). This treatment fosters the expansion of terminal airway structures without allowing these structures to collapse on expiration. A substantial proportion of patients, however, progress to MV because of inability to maintain normal arterial blood PCO_2. The long term outlook of those patients is particularly poor, and is fraught with the danger of developing bronchopulmonary dysplasia (BPD).

In addition, some premature newborns with particularly immature lungs succumb during the first few minutes of life due to inability to initially expand the lungs. It is known that preterm newborns (over 24 wks) have adequately developed anatomic air spaces which could potentially expand; those preterm infants already have a pulmonary vascular capillarity capable of pulmonary oxygen transport. These infants are unable to sustain alveolar ventilation due to instability of terminal airways. Their immaturity is reflected in very low levels of pulmonary surfactant.

We believe the technique of apneic oxygenation with extracorporeal removal of CO_2 ideal to apply in the entire patient population of preterm newborns at high risk to develop HMD, or in these newborns in whom the disease has already become established.

Methods Employed and Major Findings:

1. The carbon dioxide membrane lung is basically a spiral coiled membrane lung optimized for high CO_2 transfer. The preferred membrane until now is made of silicone rubber. More permeable microporous polypropylene membranes have over twice the CO_2 transport capability. No information is available as to performance of microporous polypropylene membrane lungs in long term use.

For infant use, the CDML has to perform well at blood flows as low as 20 - 50 ml/min. Such a requirement can now be met with a CDML we have designed based on the twin coil artificial kidney first described by Kolff. At very low blood flow, the long blood path of that design is especially well suited for the removal of CO_2, and hence for use in small newborns.

2. a. Fetal Work

The fetal lamb is the preferred animal model to study new approaches to the treatment and prevention of HMD. At approximately 130 days gestation the fetal lamb has a near 100% mortality with conventional care. Under general anesthesia we have performed C-sections in dated ewes of 131 - 138 d. gestation, and delivered the fetus but left the umbilical cord intact to effect removal of CO_2 and the supply of some oxygen. After initial insufflation of the lungs, the lungs were kept inflated at an intrapulmonary pressure of 15 cm. H_2O (apneic oxygenation) for from 4 - 6 hrs. This assured that even when the umbilical circulation slowly fell, the PCO_2 would rise only slightly; while arterial PO_2, augmented by pulmonary oxygen transport, slowly rose. The total lung compliance (TLC) gradually rose over a period of 3 - 8 hrs to levels equal those found in term newborns. The chest X-ray films gradually cleared. At that point we placed the fetuses on MV without developing pulmonary failure or HMD. All 19 animals survived for 24 hrs in excellent health. Those studies demonstrated that lungs exhibiting normal total lung compliance can be ventillated. Furthermore, those lungs continued to improve during pulmonary ventilation. Saline lung wash obtained 24 hrs after elective sacrifice remained in what is considered the abnormal range. And yet, respiratory failure (HMD) did not occur.

Unless TLC exceeds 0.5 - 0.6 ml/cm H_2O/kg, normal pulmonary ventilation is not possible and HMD is likely to develop. It appears that obtaining this TLC was the key which reflected mature lungs capable of normal pulmonary ventilation.

More recently, we have been able to accomplish pulmonary maturation faster yet in even younger, 130 d. old fetuses. By applying substantially greater intrapulmonary pressure for a few seconds only, followed by statically inflating the lungs to 15 cm. H_2O, the TLC rose to normal range much faster. In almost 80% of those fetuses the TLC became normal within 1½ hours. In 20% of fetuses gestational age of 130 days it was determined by the first one hour that a prolonged time of pulmonary conditioning was to be anticipated, the proper procedure in those cases being placement on the CDML with extracoropreal bypass. From our earlier work, such conditioning evolves over a period of 8 - 12 hrs. This accelerated pulmonary conditioning, and our ability to assess status of lung maturation, poses some interesting possibilities to the future treatment of prematures susceptible to HMD. It appears that a brief preventive intervention lasting perhaps as short as a few minutes to as long as one hour has a profound bearing as to whether or not HMD is to develop.

The value of L/S ratio is now well established as a predictive index to HMD. We believe the determination of TLC and its restoration to normal by the means described above in the face of a persistently low L/S ratio, is the simplest and a most rapid means that has yet been applied to the prevention and treatment of HMD.

b. Treatment of ARDS

These clinical studies were carried out in collaboration with the University of Milan, Italy.

Five patients with ARDS obtained immediate relief from hypoxis after beginning LFPPV with $ECCO_2R$. The pulmonary ventilation was kept at 3 - 4 breaths/min with a very low TV and a peak pressure of less than 35 cm. H_2O. Within hours of going on bypass the arterial blood PO_2 exceeded 100 mm. Hg and the F102 was lowered to 0.40. This was associated with a marked diuresis, and improvement in chest X-ray films. This rapid improvement is typical of this technique. Moreover, the ECMO criteria for discontinuing bypass (based on FIO_2 and PaO_2) were met and exceeded in less than one day. Improvement in lung compliance to permit spontaneous breathing or MV, took considerably longer, from 2 - 13 days, depending on the underlying disease pathology, related in part to the ravages imparted by mechanical ventilation.

Results so far strongly suggest that management of the lungs has great bearing on recovery of the lungs once on extracorporeal bypass. These results suggest that MV while considered at times life saving, can lead to exacerbation of the disease process, to fulminant ARDS, and death. We suggest as an alternative the use of extracorporeal CDML in the control of spontaneous breathing, and in the control of MV, and in the treatment and cure of severe ARDS.

Importance to Biomedical Engineering and the Program of the Institute:

1. Acute pulmonary failure is a life threatening process as it enters into a fullblown ARDS. We believe progression to fullblown ARDS can and should be arrested by the early application of an extracorporeal CDML in the control of spontaneous breathing, or in the control of mechanical pulmonary ventilation. This procedure is much simpler technically than high flow extracorporeal blood bypass as practiced in ECMO; more importantly, it leads to a rapid recovery of lung function. Unlike the thrice weekly use of dialysis in chronic renal failure (by way of comparison) the use of a CDML and extracorporeal bypass is a one shot procedure with no need for recurrent treatment. Once cured, always cured. The use of this procedure is to be viewed not only curative, but also

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preventive to avoid ARDS from developing in the first place.

2. Our studies in the prevention of acute respiratory failure in the preterm fetal lamb strongly suggests that HMD in man is a preventable, and a curable disease. It is exciting to learn that conditioning the fetal lung to exhibit a normal TLC can be accomplished in as short a time as in one hour. This intervention alone, and not the exogenous administration of pulmonary surfactant, appears to lead to rapid pulmonary maturation.

Such treatment can be accomplished either while still connected to the umbilical cord; after the umbilical cord has been cut, through connection to an extracorporeal CDML for a brief period of bypass.

Proposed Course:

In collaboration with Dr. Brumley (Duke University) and Dr. Ulane (NICHD) we will further explore the biochemical aspects of fetal lung maturation as found in our experimental technique. This will involve the measurement of disaturated phosphatidyl choline (DSPC) and phosphatidylglycerol (PG), and the rate of incorporation of choline into DSPC. Additional studies are planned to define the best means for rapid pulmonary conditioning using both the natural placenta, and in the younger fetuses the CDML system.

Studies in the clinical application of $ECCO_2R$ with LFPPV will continue at Milan, the HMD studies managed by Dr. Pesenti, and ARDS studied by Dr. Gattinoni. Studies on chronic hypercapneic COPD have been initiated at the University of Pisa, Italy, using the CDML in chronic extracorporeal bypass.

Publications:

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2. Gattinoni, L., Kolobow, T., Agostoni, A., Damia, G., Pelizzola, A., Rossi, G. P., Langer, M., Solca, M., Citterio, R., Pesenti, A., Fox, U., Uziel, L.: Clinical Applications of Low Frequency Positive Pressure Ventilation with Extracorporeal CO_2 Removal (LFPPV-$ECCO_2R$) in the treatment of adult respiratory distress syndrome (ARDS). Int. J. of Artif. Organs, 2: 282, 1979.

3. White, D. C., Trepman, E., Kolobow, T., Bowman, R.L.: The Blood-Membrane Bioartificial System: Hormone Production by Human Endocrine Adenomas Perfused with Sheep Blood. Trans. Am. Soc. Artif. Intern. Organs 25: 32, 1979.

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9. Gattinoni, L., Agostoni, A., Pesenti, A., Pelizzola, A., Rossi, G.P., Langer, M., Vesconi, S., Uziel, L., Fox, U., Longoni, F., Kolobow, T., Damia, G.: Treatment of acute respiratory failure with low frequency positive pressure ventilation and extracorporeal CO_2 removal. Lancet, In Press. 1980.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01405-06 LTD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Analysis of Microcirculation by Coherent Light Scattering

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	R. L. Bowman	Chief, Lab. Tech. Development	LTD	NHLBI
	P. Bowen	Biologist	LTD	NHLBI
Others :	R. Bonner	Physicist	BEI	R

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DR

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 2	OTHER: 1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The purpose of this project is the development of a non-invasive method of measuring tissue blood flow by analysis of the spectrum of doppler scattered laser light. The prototype instrument has been greatly improved by a new analysis scheme, a four meter flexible fiber optic probe, and the use of a photodiode detection system. The linearity of the instrument in response to blood flow has been greatly improved and noise has been reduced so the instrument can resolve the instantaneous pulsatile flow of microcirulation. The instrument is being used to monitor average blood flow and dynamic blood flow in normal individuals and clinical center patients. Data is being gathered to assess the instrument's usefulness as a measure of blood flow in perepheral vascular disease, carotid artery occlusive disease, sickle cell anemia, and muscular dystropy.

PHS-6040
(Rev. 10-76)

Objectives:

To continue development of techniques and applications of a method of continuously measuring the blood flow through small regions of tissue by analysis of the spectrum of coherent light which is doppler-scattered from red blood cells in the tissue of microcirculation. The instrument has been shown to be capable of indicating surface blood flow in skin, muscle, kidney, liver, spleen, brain, and gastric and intestinal wall. Research applications in vascular physiology and pharmacology have been demonstrated. Clinical applications to the study of peripheral vascular disease, burns and grafts, monitoring tissue perfusion at surgery and under the influence of anesthesia are being examined. Specific objectives at this stage are the final development of the instrument and demonstration of applications to a variety of experimental and clinical problems.

Methods:

Instrument Development

The apparatus for monitoring tissue blood flow is continuing to be improved and has been made compact and portable such that clinical and laboratory mesurements are conveniently made. Through collaboration with Dr. Robert Bonner, BEIB, we have conducted detailed analysis of the doppler spectrum and optimized the instruments linear response to blood flow in skin, kidney and muscle tissue. We have found that a more sensitive linear measure of blood flow can be obtained by calculating the normalized first moment of the Power spectrum or the mean frequency. We have incorporated a fiber optic probe into our system. This probe efficiently transmits the laser light to the subject and the back scattered light to the detection system while providing remotes flexible attachement to the patient. We have also reduced the size and expense of the instrument by using a new photodiode detection system in place of the photomultiplier. The potential and feasibility of using the new solid state Lasing diode as an alternative light source will be investigated.

Major Findings:

1. The new method of calculating flow, the normalized first moment of the power spectrum or mean frequency, appears to be a very sensitive indicator of tissue blood flow.

2. We are able to resolve the wave form of the pulsatile flow in tissue.

3. We are now able to measure flow in a wide variety of tissues. We have normalized our flow parameters to the power of the light backscattered from the tissue and have made the device relatively insensitive to changes in tissue color.

4. The fiber optic probe has greatly improved the flexibility of the instrument and facilitated remote attachment to the patient.

5. Two clinical protocol exist for studies within NIH.

We are now measuring dynamic blood flow changes in a variety of tissues including thermal reactivity, reactive hypermia and excercise hypermia as potential indicators of normal and diseased states.

We are able to assess the relative blood flow in muscle tissue. This has been demonstrated in aniaml models as well as in exposed human muscle during biopsy. The instrument has been used to measure supraorbital blood flow as a possible indicator of altered flow in carotid artery occlusive disease. These studies could lead to conclusions on the relationship of blood flow to abnormal states.

Significance to Biomedical Research and the Program of the Institute:

Laser doppler spectroscopy is a promising method of studying microcirculation of tissues.

It has potential applications not only in the laboratory, but in the clinical study of the peripheral vascular disease, the screening of vasoactive drugs, and the monitoring of patients with unstable circulatory systems.

Proposed Course:

1. Continue development of the instrument in collaboration with BEIB or industry.

2. Cooperate in clinical trials to establish the instrument as a useful clinical dianostic tool.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01407-17 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Luminescence Spectroscopy in Biomedical Research

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Raymond F. Chen	Senior Investigator	LTD	NHLBI
	Robert Meeks	Postdoctoral Fellow	LCB	HCI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Luminescence methods, especially fluorescence spectroscopy, are uniquely suited for studying certain biomedical problems. The areas investigated were: 1. The activation and inhibition of phospholipase activity, as studied by a dye-liposome fluorescence assay. 2. The toxicity of retinoic acid analogs, as studied by fluorescence polarization of a membrane probe. 3. Energy transfer between different sites on serum albumin. 4. The kinetics of labeling of proteins and amino acids by the o-phthaldialdehyde reagent.

PHS-6040
(Rev. 10-76)

Objectives: The purpose of the project is to apply fluorescence methods to problems of interest in biomedical research, thus demonstrating the usefulness of some of the methods which were developed in this laboratory, and stimulating further methodological developments. In so doing, problems of intrinsic interest are elucidated.

Methods Employed: Chemical specimens were purified in the laboratory or supplied by collaborators. The fluorescence measurements of quantum yield, spectra, lifetimes, and polarization were performed on modified commercial instruments. Stopped-flow kinetics were performed with units designed with the aid of Dr. Berger of this laboratory. Computer calculations were done on the PDP-10 computer.

Major Findings:

1. The program to investigate membrane effects of retinoids in collaboration with Dr. Meeks, LCP/NCI, was continued. Rat erythrocyte ghosts were labeled with a membrane probe, diphenylhexatriene (DPH). The DPH fluorescence polarization is inversely proportional to membrane fluidity, and it was found that there was a good correlation between the effectiveness of retinoids in increasing fluidity, and their toxicities. Light scattering experiments showed that, although membrane fluidity was dramatically increased by retinoids, there was no corresponding change in light scattering, as one would expect if the membrane was being dissolved by a detergent-like material. A detailed report of these findings has been submitted, and another paper dealing with the correlation between toxicity and probe depolarization also has been submitted.

2. Serum albumins are important transport and osmotic pressure-maintaining blood proteins whose structures have been under study for many years. Human serum albumin contains about 580 amino acids in a single chain, whose sequence is now known. However, the three-dimensional structure has not yet been elucidated by X-ray crystallography. There are several sites of interest in the protein: 1 - The site of the single tryptophan residue. 2 - There is a single free sulfhydryl group. 3 - There is a site for binding billirubin. 4 - There are additional sites for binding fatty acids, metal ions, etc. These sites are not identical, and it is possible to investigate the distance between them using the technique of fluorescence energy transfer. We have approached the problem by using tryptophan as energy donor, and attaching various dyes to the free sulfhydryl group as acceptor. Using Forster's theory of resonance energy transfer, we estimate the distance between the sites to be about 24 Angstroms. The distance between the sulfhydryl group and the billirubin site is being investigated using the attached dye on the sulfhydryl as donor, and bilirubin as acceptor. A variety of dyes will be used to rule out untoward idiosyncrasies of any given dye.

3. We have continued the study of phospholipase activity and related phenomena using the assay system consisting of liposomes containing a concentrated dye solution. The dye, 6-carboxyfluorescein (6CF), is largely non-fluorescent until released, due to concentration quenching. Spectral analysis done here

suggests that the concentration quenching is due to dimer formation and energy transfer to the dimers. When the lecithin liposomes are subjected to hydrolysis by phospholipases, fluorescence is markedly enhanced thus providing a convenient assay of activity. Stopped-flow measurements where dye-liposomes are mixed with ethanol show that the fluorescence increase is instantaneous, and not limited by diffusion. It has also been found that phospholipase C and pancreatic phospholipase A_2 are inhibited by serum albumin, while the liposome lysis by snake venom phospholipases is enhanced by serum albumin. There are interesting mechanistic and teleological implications of these findings. Mellitin, which is a polypeptide found in bee venom, is very effective in lysing the liposomes. Less than 1 mellitin molecule per 100 lecithin molecules results in essentially complete release of dye.

One paper has been prepared and is currently under review.

4. We have continued work on our observation that certain enzymes are activated or inhibited by phosphatidylcholine, or lecithin. Since lecithin is the major component of most biological membranes, this observation may be relevant to mechanisms of control over enzyme activity. We have supplemented the previous observations by examining the effects of a series of phosphatidylcholines of different chain length, as well as other phospholipids. The actual association of enzymes such as alcohol and glutamate dehydrogenases with lipids has shown by measuring the intrinsic and extrinsic fluorescence of the enzymes as a function of added lipid. These results will shortly be ready for inclusion in a paper.

5. Using stopped-flow kinetic measurements, we have extended previous observations on the rate of reaction of o-phthaldialdehyde with various amines. Previously we had measured the rate of reaction with a number of amino acids, and, with Elly Trepman, have submitted a paper with those results. Now we have looked at the reaction rate with some di-amines and proteins. The diamines react in two steps, which can be resolved since the second fluorescing group quenches. The proteins react with different rates, reflecting the different number and accessibility of reactive groups. Analysis of such data will hopefully provide information on accessibility and conformational changes brought about by different agents.

Significance to Biomedical Research and the Program of the Institute:

The present studies advance the basic understanding of certain biological systems of importance to medicine; e.g., Vitamin A and its toxicity, the action of phospholipases, and the structure of human serum albumin. Such studies are consistent with the policy of the institute in furthering elucidation of biological processes.

Proposed Course: Much of the work described has already been included in papers which are under review; the completion of the publication process will be of high priority. We intend to continue work on the energy transfer project, which is currently yielding good results. The stopped-flow kinetics on labeling of proteins with fluorescent dyes will be continued, as this seems to be a unique method for studying macromolecular structure.

Publications:

1. Trepman, E. and Chen, R.F.: "Fluorescence Stopped-Flow Study of the o-Phthaldialdehyde Reaction", Arch. Biochem. Biophysics, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01408-15 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Methodology in Fluorescence Measurements

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Raymond F. Chen	LTD	NHLBI

COOPERATING UNITS (if any)

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NIH, NHLBI, Bethesda, Maryland 20205

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SUMMARY OF WORK (200 words or less - underline keywords)

1. The purity of human serum albumin has been studied. Commercial preparations differ in their fluorescence spectra, quantum yields, and fluorescence decay parameters. Removal of impurities alters these properties. These findings are relevant to the use of human serum albumin in optical studies.

2. Fluorescence depolarization measurements have commonly been made on proteins to determine their size and shape. We have investigated the use of the proteins' native ultraviolet fluorescence for this purpose instead of the fluorescence of attached dyes. The results suggest that the use of dyes is not always necessary, reasonable results can be obtained with the intrinsic fluorescence, although caution must be taken to factor out the independent rotary motion of the indole groups.

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PHS-6040
(Rev. 10-76)

Objectives: The purpose of the project is to develop methods relating to fluorescence spectroscopy as applied to biomedical research. In this way we enhance the usefulness of fluorescence.

Methods: The instrumentation used to obtain fluorescence parameters are those which have been developed in this laboratory over the years. In addition, we have collaborated with Dr. Vaughn Koester of the Physiology Department, University of Texas Health Science Center, Dallas, Texas, who has made fluorescence decay measurements with a picosecond, frequency doubled, tunable, synchronously pumped dye laser system.

Major Findings:

1. The fluorescence characteristics of 12 commercial samples of human serum albumin were examined before and after treatment to remove bound impurities. The emission spectra of the untreated samples varied in shape, and the relative quantum yields varied widely (standard deviation from the mean was 26.4%). Dialysis and charcoal treatment (Chen, 1967) minimized the spectral differences. The quantum yields still varied (15.3% standard deviation from the mean). The observations could be explained by removal of fluorescent impurities from some samples and nonfluorescent (but quenching) impurities from other samples. The fluorescence decay kinetics, using the picosecond flash method, clearly showed differences between the samples. Even crystalline samples of human serum albumin differed in properties. The paper containing these observations contains a caution that, as a minimum, optical studies with human serum albumin should be proceded by removal of impurities by charcoal and dialysis treatment.

2. Our previous work with Dr. Oliver Alabaster, NCI, which demonstrated and explained an artifact in the Los Alamos cell sorter has been written up and is in press. The work showed that fading in the instrument was due to electrolytic evolution of chlorine, which caused bleaching of the supravital dye.

3. Fluorescence polarization is related to the Brownian rotational mobility of a fluorescent substance. The method was developed by G. Weber to determine the rotational properties of proteins to which dyes were attached. Normally, the native, or intrinsic, fluorescence of the proteins due to trytophan groups is not used, because the fluorescence lifetime is usually too short. With the advent of more accurate measurements, polarization and lifetime measurement using the intrinsic fluorescence seem feasible. If the polarization of tryptophenyl fluorescence is measured, it can be seen that the tryptophan must have independent mobility superimposed on the rotational mobility of the proteins as a whole. To separate these two mobilities, we have first measured the polarization of the proteins in glycerol at low temperatures; here the protein is essentially immobilized, but tryptophan still rotates within the protein interior. The amount of depolarization due to the protein can then be inferred. The advantage of this method of studying protein size and shape is that the need for an added dye is abolished, and the tryptophan(s) are randomly oriented with respect to the protein axis.

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Significance to Biomedical Research and the Program of the Institute:

The first spectrofluorometer was developed in this institute some 25 years ago, and the laboratory has continued the tradition of fluorescence by developing methods which make the technique more useful. Our work, which deals with purity of proteins for fluorescence work, elucidation of artifacts in the fluorescence flow cell sorter, and the fluorescence depolarization of proteins, carries on that tradition.

Proposed Course: The depolarization data already obtained can be completed in a short time and will show how to separate out various rotational motions in proteins. The purity of serum albumins is of interest to many people, and we already have much data on bovine and other species' albumins in addition to that obtained for human serum albumin. This work can be extended.

Publications:

1. Chen, R.F. and Koester, V.J.: Fluorescence Properties of Human Serum Albumin: Effect of Dialysis and Charcoal Treatment, Analytical Biochemistry in press.

2. Alabaster, O., Glaubiger, D.L., Hamilton, V.T., Bentley, S.A., Shackney, S.E., Skramstad, K.S. and Chen, R.F.: "Electrolytic Degradation of DNA Fluorochromes During Flow Cytometric Measurement of Electronic Cell Volume", J. Histochem. Cytochem. 28, 330-334, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01411-14 LTD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Blood Flow Measurements Using Nuclear Magnetic Resonance Techniques

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	V. Kudravcev	Engineer	LTD	NHLBI
	R. L. Bowman	Chief, LTD	LTD	NHLBI
	J. Pochobradsky	Expert	LTD	NHLBI
Others:	A. Sances	Professor and Chairman Biomedical Engineering	Medical College Wis.	
	J. H. Battocletti	Associate Professor Dept. of Neurosurgery	Medical College Wis.	

COOPERATING UNITS (if any)

Medical College of Wisconsin, Milwaukee, Wisconsin

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 3	PROFESSIONAL: 3	OTHER:

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☒ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Nuclear magnetic resonance flow methods have been developed to measure blood flow in perepheral vessels using magnetic markers for venous flow or self tag methods for pulsatile flow. Specific vessels are tuned in by a "ranging" method whereby a sensitive region is localized by the interaction of external magnetic fields.

Several NMR signal enhancement systems are being investigated to increase the sensitivity of the method based on observations of double resonance phenomena whereby short relaxation time materials i.e. rubber surrounding the proton signal sample, couple with the flow proton signal to improve the S/N ratio. Similar systems utilizing NMR coupled to electron spin resonance are also being investigated.

Flow signals from specific blood vessels within the brain have been localized by a "ranging" method and detected by the self tag method.

Detection systems at high, low, and weak fields have been set up to study the time limits of detectability of proton magnetic markers in blood.

PHS-6040
(Rev. 10-76)

Project Description

The objective of this project is to develop a system that can measure changes in cerebral blood flow useful to the problem of determining who is at risk for stroke or circulatory limitations of function. The system has the potential to measure cerebral perfusion and changes in perfusion in specific selectable portions of the brain without including flow in overlying structures. No injection or radioactivity is used and all the modalities used are harmless and can be repeated as often as needed.

Methods Employed

As a continuing project several systems are being investigated to select the most practical one. Protons in the blood are easily detected by NMR methods and variations in their magnetic properties produced by flow form the basis for flow measurements. A relatively slow relaxation time of proton induction is the basis for dynamic marking by magnetic fields.

Several methods of flow measurement have been developed and published using these dynamic markers either induced or passive and demonstrated to be effective and sensitive for peripheral blood flow but effective measurements localized to an internal portion of the brain in the intact human head have just recently been accomplished.

The demonstration was accomplished by our contract facility at the Medical College of Wisconsin where the installation of a large superconducting magnet provides the large magnetic field necessary for these experiemnts.

The method utilizes detection by systems of projected fields derived from flat crossed coil configurations developed over the past project years and the development of a "null" field region to localize the sensitive volume in a spce that is projected inside the head to limit the detection conditions to the locale of a specific artery.

Signals and measurements have been demonstrated from the middle cerebral artery and also demonstrated for vessels over the heart, and vessels in the leg. Some preliminary measurements on patients confirmed known clinical blood flow deficiency with the in depth ranging method.

In a continuing effort to use a system based on premagnetized blood being demagnetized in a specific cerebral volume to evaluate the regional flow we are investigating various signal enhancement ideas which should make it possible to discriminate the small signal changes that would persist after the relatively long transit time through the brain.

The signal enhancement systems are based on double resonance effects that have been noted when two resonating systems couple to effectively filter or narrow the bandwidth of the signal and effectively increase the detectability or signal-to-noise ratio.

Efforts to couple the short relaxation of rubber protons to the protons in the flowing stream have demonstrated significant enhancement and suggest the possibility of similar but greater enhancement of the electron spin resonance of a paramagnetic sample were coupled to the proton resonance. In the ESR system a magnetic field of 23.4 gauss, the ESR frequency is about 65 mHz and the NMR frequencies is 100 KHz.

Efforts to obtain enhancement indicated deficiences in the electronics which will be corrected.

In addition further exploration of the limitations imposed by the short relaxation time of blood at low fields and frequencies due to the interaciton of protein dipoles will be explored to find either a frequency "window" or sufficient signal from free protons that do not interact with the dipoles.

Significance to Biomedical Research and the Program of the Institute:

The need for a harmless method for studying the status of the cerebral circulation in the apparently healthy individual has precluded any studies of the early course of cerebral atherosclerosis or other changes leading to cerebral atrophy. In addition any early therapy or environmental change could not be followed over a long time course without a very low morbidity hazard.

Proposed Course:

In LTD the development of signal enhancement and sensitivity methods will be continued. The contract program at the Medical College of Wisconsin has completed the development of a concept and method for cerebral flow by the ranging technique. Delays in apparatus and supplies limited the work so that the contract while complete in concept has in no way become non-productive.

As the contracts with Medical College of Wisconsin have assembled a scientific staff, superconducting magnet facilities, and an interaction with neurosurgical and nuerophysiological facilities for further studies, the laboratory plans to recommend intramural contract support for the continued development of the system and for the conduct of tests of the effectiveness of the method for measurements of intracerebral blood flow in humans and primates.

Publications:

1. Sales-Cunha, S. X., Halbach, R. E., Battocletti, H. J., Towne, J. B., and Sances, A., Jr. Noninvasive techniques in the evaluation of the peripheral circulation. J. of Clinical Eng., 4(3):209-220, July-Sept., 1979.

2. Sales-Cunha, S. X., Halbach, R. E., Battocletti, J. H., Kauffman, H. M., Adams, M.B., and Sances, A., Jr.: Noninvasive flowmetry in upper limbs of patients with arteriovenous dialysis fistulas. IEEE 1979 Frontiers of Engineering in Health Care, Denver, Colorado, Oct. 6-7, 1979, pp 175-179.

3. Battocletti, J. H., Halbach, R. E., Salles-Cunha, S. X., Sances, A., Jr., Towne, J. B., Hebert, L.A., and Kauffman, H. M.: Clinical applications of the NMR limb blood flowmeter. Proc. IEEE (Special issue on Technology and Health Care), 67(9): 1359-1361, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 HL 01413-18 LTD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Instrumentation for the Study of Pre-Steady State Enzyme Kinetics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	R. L. Berger	Chief, Biophys. Inst. Section	LTD	NHLBI
	P. D. Smith	Expert	LTD	NHLBI
	G. w. Liesegang	Staff Fellow	LTD	NHLBI
	P. Bungay		BEIB	DRS
Others:	J. Froelich		Nat. Ageing Inst.	
	C. Gibson	Electrical Engineer	BEIB	DRS
	W. Friauf	Chief, Electrical Eng. Sect.	BEIB	DRS
	W. Hurst			NBS
	H. Cascio	Electrical Engineer	BEIB	DRS

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS, NIH

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Biophysical Instrumentation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
4	3	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An improved ball mixer has been developed which will hopefully reduce the mixing heat artifact by at least a factor of ten. Thin film sensor development has continued with theoretical models set up to test potential barrier designs which should greatly improve the stability and reproducibility of these devices. A new observation chamber has been constructed for the high speed stopped-flow apparatus. After several hundred experiments it still does not leak. Fiber optic light guides have been used to eliminate the optical distortion produced at stopping due to vibration. Ca+EGTA and CO + Hemoglobin have been used as test reactions. A dual wavelength, 660 and 900 nm detection head for the laser flash photolysis apparatus has been put into operation. Identical rate constants were found for 7mM (in Fe) HbCO at both 660 and 900nm $[1.6 + 10^5 M^{-1} sec^{-1}]$ in a 2mm optical path. Flash was 1J at 580nm. The reaction of Ca(+2) or Li(+1) with Cryptand 211 has been studied using the thermal stopped flow apparatus. Results indicate a fast bi-molecular initial binding step flowed by a slower isomerization reaction. Both rates and ΔH's are measured simultaneously.

Project Description

Objectives:

The objectives of this project are to develop new instrumentation methods, data handling techniques and theoretical treatments for the physiochemical study of the thermodynamics, kinetics and thus the mechanisms of enzyme action in solution and in the intact cell or cell membrane. In particualr, to develop methods and instruments to study, in collaboration with other laboratories, the reactions of hemoglobin with the respiratory gases both in normal state as modified by the changes of physical factors, small molecules, various metabolites, and genetically, such as in sickle cell anemia. The reactions of various cellular enzymes, particularly ATPase, lactate dehydrogenase, and 2-3DPG and their interactions and control in the cell are studied as they relate to the hemoglobin reactions in cardiology, pulmonary and respiratory function, and circulation. Where appropriate analytical methods are developed for research and clinical application.

Methods Employed:

The methods used in the investigation of the mechanisms of enzyme action are those of pre-steady state chemical kinetics and thermodynamics. Measurements of the appropriate parameters are made by developing the necessary equipment to mix solutions rapidly and follow the course of the resulting chemical reaction by optical, thermal, glass electrode, etc., detectors. In general, equipment is not available, either in the literature or commercially, for investigations in this area. Such apparatus is conceived and designed in this laboratory, together with consultants, construction being carried out wherever most appropriate; i.e., in our shops or by commercial firms, special university facilities, or at several special research laboratories such as the Jet Populsion Laboratory. In pursuing these investigations, a wide variety of physical parameters must be studied, which leads to the need for an understanding of the underlying physical theory governing the reactions. Expert consultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment, particularly as it is applied to certain specific enzyme systems under investigation.

Major Findings:

Flow

A substantial improvement to the high speed stopped flow apparatus has been achieved. A leakage of reactants from the apparatus through the observation window to observation chamber seal has been eliminated. This problem was a serious drawback to routine operation of the apparatus since the leakage was in the optical absorption path. A complete redesign of the observation window to chamber seal was performed with highly successful results.

The flash photolysis apparatus has been used in conjunction with a high speed dual wavelength detector to observe the kinetics of carbon monoxide binding to hemoglobin at a concentration of 7mM. The detector has two observation wavelengths at 660 nm and 900 nm and consists of a pair of LED's which are detected by an integrated photodetector/amplifier package (EG&G HAD 1000). Recombination kinetics have been observed with a rate constant of $1.2 \times 10^5\ M^{-1}\ s^{-1}$ in agreement with published results.

A thermistor modification to the Aminco-Morrow stopped flow apparatus has been completed. A 7 msec. response time thermistor was incorporated into the Aminco-Morrow with a minor modification to the observation chamber. Chemical kinetics with half lives to 15 msec. have been measured with a sensitivity of 1m°C. A test reaction of 1M $NaHCO_3$ to .05M HCl was performed and compared favorably with the optical curve.

The thermal stopped flow (TSF) was tested for its suitability as a monitor of ion-ligand (e.g. protein) interactions. A test chemical system was needed which fulfilled several requirements: i) the ligand molecule had to exhibit complexation properties similiar to the metallo-protein interactions; ii) the kinetics of complexation must follow the general reaction scheme $A+B \rightleftarrows C \rightleftarrows D$; iii) the relaxation (rate) has to occur within the stopped-flow time regime; and iv) at least one reaction have a small ΔH (<1kcal/mole).

A class of compounds known as synthetic multidentate ligands fulfill all of the above requirements and, within this class, the cryptands are one of the more interesting species. Cryptands are two-ringed macrocycles containing both nitrogen and oxygen atoms (with bridge head nitrogen atoms). These ligands form stable complexes since the complexed ion is contained within the central molecular cavity of the macrobicyclic ligand. These cryptands also exhibit a remarkable selectivity based upon the radius of the ion, however very little is known about the mechanism of ion-complexation, due to a lack of a suitable probe available with which to monitor the extent of reaction. For these reasons it was felt this would be a suitable system with which to test the TSF.

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The binding of lithium(+1) and calcium(+2) ions to the cryptand 211(4,7,13,18-tetraoxa-1,10-diozabicyclo(8,5,5) eicosane) as investigated in aqueous media at 25.0° C. The solutions were buffered at pH 11.4 (piperidine-HCl buffer system), where effects from the acid-catalyzed decomplexation is minimal, and a high percentage concentration of the cryptand is in the unprotonated form. The ionic strength was maintained (with buffer) at 0.1M. Concentration ranges of metal ion and cryptand were from 3.5-50mM, after mixing. Over this concentration range both Li(+1) and Ca(+2) exhibited a minimum of two relaxations. For Li(+1) complexation, a fast reaction occurred within the dead time of the appartus, followed by a slower and measurable heat change. In the case of Ca(+2) there is a biphasic reaction occurring after stopping with near equal but opposite heats. For both ions no reaction was observed when either metal ion or 211 was absent. A test for a concentration dependence revealed that (for both ions) the slower reaction was strictly first-order, while for Ca(+2) the faster (endothermic) reaction best fitted a second-order concentration dependency. It would appear therefore that a possible mechanism is:

$$M^{N+} + L \rightleftarrows (M^{N+}L)^{1} \rightleftarrows (ML)^{N+} \qquad (1)$$

Reaction scheme 1 is similar to that proposed by others, but includes a subsequent conformational rearrangement of the ligand after ion-complexation.

A thorough analysis of both the kinetic and thermodynamic parameters was undertaken. We were able to evaluate the rate constants k_1 and k_{-1}; the enthalpic heats of each individual reaction step ΔH_1 and ΔH_2 and the overall heat of complexation H; the equilibrium constants K_1 (= k_1/k_{-1}) and $K_2 = (k_2/k_{-2})$; and the entropies of reaction ΔS_1 and ΔS_2. Where possible, comparisons were made between our values and literature.

Finallly it should be noted that during this study several improvements were made to the thermal stopped flow. In addition several areas of improvement such as use of a DC bridge, "real-time" signal averaging, and an improved ball mixer have been developed and are undergoing tests. This unit should greatly reduce the heating artifact by a factor of ten, and allow much higher flow relocation in the quench flow apparatus before damage occurs to the sacroplasm reticulum.

Thin Film Temperature Sensors

Work has begun on fabrication of capacitive M-I-S structures on silicon material in which deep donor levels have been established. These levels will be created by introducing impurities into the silicon by means of ion beam implantation. The impurity material will be chosen such that the system Fermi level lies above the deep impurity level. If the positions of the impurity and Fermi levels are properly established, the capacitance of the barrier region becomes highly temperature sensitive.

The I-layer of the structure will be produced by thermal oxidation.

The Si material obtained for these studies is N-type, of 1-1-1 orientation, with a low 10^{14} cm^{-3} impurity level. The wafers were polished at N.B.S. using the facilities of the semiconductor processing group. These facilities use standard polishing and cleaning techniques, and the system is monitored periodically by manufacture of test devices. After cleaning and dicing into 1 cm squares, samples were implanted with impurities of magnesium, tellurium, neodymium, or thulium. These impurities were chosen since they only exhibit a single impurity level and they cover a convenient range of 0.1 to 0.3 eV below the conduction band. Implants were done with total influences of 10^{12} to 10^{16}, and peak densities of 10^{17} to 10^{21} cm^{-3}. Ohmic contacts were then placed on the back side of the wafers. This proved to be somewhat of a problem, since the Si is high-resistivity material. Low resistance ohmic contacts were finally produced by first doing low-temperature drift of a boron solution and then coating with aluminum. (High temperature drifts for ohmic coating would interfere with the temperature-drift studies that must be done for annealing and drifting the ion-implanted impurities.) In the future, ohmic contacts will be established on the back before ion implanting. The samples are now ready for annealing studies at temperatures between 800 and 1100 deg. C.

In addition, we have located some special-purpose commercial Si that is indium-doped. This material is p-type, but also possesses shallow acceptor impurity levels. These have been carefully compensated for by the introduction of corresponding shallow donor levels. Capacitance test patterns are now being placed on this material for testing as a possible temperature sensor.

The modeling of the physical characteristics of the barrier region has been extended. A complete derivation will not be given here. It is interesting to note that the temperature sensitivity of the barrier capacitance is independent of the impurity dopant density. (However, the total capacitance of the device varies as the square root of the dopant density.) Curves of the temperature coefficient of the barrier capacitance, alpha, versus the potential of the barrier height at the semiconducting surface, psi, have been determined. The quantity (E-V) is the position of the Fermi surface below the conduction band.

Work on fabrication of ZrO_2 M-I-M capacitance structures in a new planar geometry is proceeding. The new mask geometry has been designed and the masks fabricated. Deposition of the sensors onto the substrates will occur at the first oportunity for use of the evaporator.

Proposed Future Work

The fast thermal stopped-flow apparatus will be rebuilt into a small more compact and easier to use unit. Particular emphasis will be put upon optimization of the design for performance, ability and ease of operation by the biochemists, computer coupled control, data acquisition, correction and model testing on line. Work will continue on a new multiple mixing, continuous flow system to be used with acid and low temperature quenching and filtration. Completion of the laser flash dual wavelength system will be carried out.

Significance to Biomedical Research and the Program of the Institute

The study of the kinetics of chemical and enzyme-substrate reactions is a widely applied field yielding important information for interpreting the mode of action of the chemical or enzyme under study. Continued development of the response and the sensitivity of different sensors and techniques, eg. thermal, pH, and optical methods, enhances the ability of an investigator to probe with finer detail a system of interest. The particular reactions chosen to test the capability of the instrumentation being developed are of immediate significance: binding of calcium by EGTA is important for studying muscle contraction kinetics, studying hemoglobin-ligand binding studies provide information for understanding the complex action of this molecule; the cryptand study is a fine example of a reaction which can only be observed by thermal methods.

Publications:

1. Bowen, P., Balko, B., Blevens, K., Berger, R. L., and Hopkins, H. P., Jr.: Stopped flow microcalorimetry without adiabatic compression: Application to reactions with Half-lives between 3 and 50 ms. Analytical Biochem. 102, 434-440, 1980.

2. Malyj, M., Smith, P.D., Balko, B., and Berger, R.: Thermal kinetics using a modified commercial stopped flow apparatus. Rev. Sci. Inst. 1980, in press.

3. Balko, B., Bowen, P., Berger, R. L., and Anderson, K.: Fast Stopped-Flow Microcalorimeter, Biophysical and Biochemical Methods, in press, 1980.

999

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF
HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE
NOTICE OF
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER
Z01 HL 01414-08 LTD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Development of Microcalorimeters and Differential pH Thermal Titration Appratus for Biochemical Reaction Studies

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	R. L. Berger	Chief, Biophys. Instrum. Sect.	LTD NHLBI
Others :	J. Everse	Prof. of Biochemistry	Texas Tech. Univ.
	H. Hopkins	Prof. of Chemistry	Georgia State Univ.
	C. Mudd	Mechanical Eng.	BEIB DRS
	W. Friauf	Chief, Elec. Eng. Sect.	BEIB DRS
	T. Clem	Chief, Elec. Eng. Sect.	BEIB DRS
	M. Sapoff	Pres., Elec. Eng.	Thermometrics, Inc.
	N. Davids	Prof. Emeritus	Penn State Univ.
	R. Winslow	Chief, Red Cell Disease Branch	CDC

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, NIH
Thermometrics, Inc., Edison, N.J.

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	2	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The differential batch calorimeter has been redesigned to eliminate cross-heating between cells. Improved thermostating has permitted a long term stability (i.e. 24 hours) of ± .2 µwatts to be achieved. The rotational artifact is less than 4 µjoules. A second generation digital thermometer has been constructed and is undergoing tests. A new thermistor probe has been designed to go with it so that the absolute temperature in a 1 cm reaction cell can be measured to ± 0.01°C referenced to the International Practical Temperature Scale of 1968 (IPTS-68). The differental pH - thermal titration apparatus has been automated under computer control both for running the experiment and collecting the data.

PHS-6040
(Rev. 10-76)

Objectives:

An understanding of the fundamental mechanism of biological reactions requries three physical factors a) the kinetics, i.e. pathway of the chemical reaction, b) the thermodynamics of the reaction, i.e., what energy is available and how does it change from one state to another and c) the stereochemistry of the reactants. This project addresses b. By developing appropriate instrumentation to study heat changes a deeper insight into the mechanism of the relevant biochemical reactions involved in the functions of enzymes and cells will be obtained. Specifically the oxygen and CO_2 reactions with hemoglobin are studied.

Methods Employed:

Initial designs are constructed in this laboratory with special assistance from commercial firms in the construction of sensors; contracts are let, where warranted, for the development of completed instruments with refinements that would tax our own facilities. The instrument is then tested in conjunction with other interested biochemical calorimetrists utilizing appropriate enzymatic and cellular reactions.

Major Findings:

The differential batch microcalorimeter has been redesigned to eliminate cross heating from one cell to the other. New test heaters and a controlled power pulse generator has been constructed and tested. This unit generates the seven test pulses agreed upon by the Cadarache Thermokinetics International Conference for testing mathematical methods of analyzing the output of calorimeters. Analysis is presently being carried out by three different methods including the Finite Element Simulation Technique (FEST). Considerable effort has also been expended in analyzing the heat distribution in microcalorimeters by several methods with particular emphasis on FEST and the method of Laplace transforms.

The differential pH-thermal apparatus has been automated by utilizing a microcomputer to drive the reactants, sense rate of chage of pH and adjust delivery rate to keep pH change within present limits per second. Multichannel data collection is checked. Thermal data correction is then carried out and a special FEST program used in conjunction with the curve fitting part of MLAB to obtain the pH's and Δ Hi's. All of this is done on the microcomputer.

A new digital thermistor linearizer has been constructed which is small enough to sit on the laboratory bench. It only samples the temperature at one or more second time intervals but it does compute the standard deviation and mean temperature of 100 samples if desired. A new thermistor small enough to go in a 1 cm cuvette, i.e. 1.5 mm in diameter, has been constructed and will soon undergo long term stability tests. It is hoped this will supply a long needed laboratory temperature standard referenced to NBS (IPTS-68). Work has continued on the glucose monitor with a new immobilizing method having been achieved which appears to have long term stability in isotonic saline solution. A new digital bridge is being designed to utilize these devices and give the needed long term stability and accuracy required for in vivo glucose mesurements.

Proposed Course:

A new differential calorimeter will be tested and applied to a number of biochemical reactions. In addition an attempt will be made to operate it in a scanning mode to study the intra-molecular forces in the folding of proteins. Work will continue on the flow microcalorimeter and the Differential pH apparatus. The digital thermometer will be given full tests at both NBS and CDC.

Significance to Biomedical Research and the Program of the Institute:

An understanding of the basic mechanisms by which enzyme catalyzed reactions carry out the vital processes of life is of fundamental importance. A necessary part of this understanding is provided by a measurement of the thermodynamics of the reaction. This will ultimately lead to enhancement of our ability to prevent and treat the heart, lung, and blood ills the Institute is charged with.

Publications:

1. Marini, M. A., Martin, C. J., and Berger, R. L.: Calorimetric Determination of the Enthalpy of Ionization of Oxidized and Reduced Horse Heart Cytochrome c. Biopolymers, Vol. 19, 899-911, 1980.

2. Marini, M. A., Marti, G. E., Berger, R. L., and Martin, C. J.: Potentiometric Titration Curves of Oxidized and Reduced Horse Heart Cytochrome c. Biopolymers, Vol. 19, 885-898, 1980.

3. Berger, R. L., et. al.: A Digitally Linearized Thermistor Thermometer Referenced to IPTS-68, Clin. Chem., in press.

4. Marini, M. A., Martin, C. J., and Berger, R. L.: An Evaluation of the Average Heats of Ionization of Reduced and Oxidized Horse Heart Cytochrome c. Anal. Letters 12(B11), 1137-1147, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01415-07 LTD

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Italy - U. S. Cooperative Science Program - Blood Gas Instruments, Project 78

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I. :	R. L. Berger	Chief, Biophys. Inst. Section	LTD	NHLBI
	L Rossi Bernardi	Professor of Biochemistry	U. of Milan	
	R. Winslow	Chief, Red Cell Diseases		CDC
	C. Monge	Senior Fogarty Scholar	FIC	NIH
Others:	M. Luzzana	Dir. Clinical Computer Science	U. of Milan	
	C. Gibson	Electrical Engineer	BEIB	DRS
	L. Thiabault	Mechanical Engineer	BEIB	DRS
	G. Dossi	Electrical Engineer	U. of Milan	
	H. Cassio	Electrical Engineer	BEIB	DRS
	H. Hopkins	Professor of Chemistry	Georgia State U.	

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, DRS, NIH
University of Milan, School of Medicine, Milan, Italy

LAB/BRANCH

Laboratory of Technical Development

SECTION

Section on Biophysical Instrumentation

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3	1.5	1.5

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new OEC cell has been constructed using improved temperature controls. Work has also progressed on the construction of a diffusion membrane system that would permit both concentrated Hb solutions and whole blood to be measured either from oxygenated to deoxygenated or the reverse. The effect of high hematocrit on the measured blood pH has been carefully examined and found to be due to the effect of the charge on the red cell on the glass electrode and not the reference junction potential. A complete physiochemical analysis of the glucose-hexokinase system has been carried out in order to explain the experimental results obtained with the differential pH apparatus.

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Objectives:

An understanding of the various physical factors that affect the delivery of oxygen to the tissues and the removal of CO_2 are vital to a correct interpretation of the physiological response of the human body to various disorders, disease states, and environmental stress. As part of a long range basic research program being carried out in collaboration with the School of Medicine, University of Milan, the Hematology Division, Bureau of Laboratories, CDC, and the Cero dePasco High Altitude Research Laboratory, Universidad Peruana Cayetaho Heredia, Lima, Peru, we are engaged in the development of highly accurate instrumentation to measure the oxygen dissociation curve in whole blood and concentrated hemoglobin solutions, the hemoglobin components, in whole blood, Hb, Hb+, HbCO, and HbO_2, the pH, pCO_2, and PO_2, and the concentration of 2-3DPG. Ultimately we hope to measure the tissue pO_2 and PCO_2 levels at the mitochrondia cytochrome oxidase site.

Methods Employed:

A systematic analysis of the complex interrelationships among several variables and the effect on the oxygen dissociation curve requires the development of a method to obtain oxygen dissociation curves of human blood in vitro, under conditions closely simulating the in vivo situation of the patient. Instruments are developed either at NIH and/or Milan, tests on pure hemoglobin are generally conducted first in Milan, where a large group is currently working on the purification of hemoglobin.

Testing on patient blood is then carried out at the Red Cell Disease Branch, CDC and at Cero de Pasco, Peru. Close cooperation exists with the medical school hospital in Milan where on-line computer monitoring will be carried out using the membrane oxygenator system developed in this laboratory by Dr. Theodor Kolobow.

Major Findings:

A new oxygen equilibrium curve appratus (OECA TM) cell has been constructed to accommodate greatly superior oxygen and carbon dioxide electrodes and give improved temperature control. In addition, the cell has been modified so that it can use a membrane for gas exchange thus permitting use in concentrated hemoglobin solutions as well as whole blood. This new method will be under complete microprocessor control with on line data collection and analysis. A detailed study has been carred out on the affect of the charge on the red cell on the glass electrode in an attempt to explain the 0.2 pH lower reading obtained on whole blood of patients with hematocrits over 75. Using cation resins it has been found that the effect is entirely due to the reaction at the

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glass electrode surface and not at the reference junction as was previously thought. The coating of the glass electrode with Lycra almost entirely eliminated this effect by keeping the resin, or red cells, away from the surface but permitting the H^+ ions to pass through.

The differential pH apparatus has been improved by greatly improving the stability of the differential pH meter. Long time stability of ± .00005 pH units is now routine. One of the simpler test systems we have used is the glucose - hexokinase system. This system has now been completely analyzed from a physical chemical standpoint to take acount of pH shifts due to Mg, ATP, and NaCl. Detailed tests are presently being performed to verify theory by experiment.

Significance to the Program of the Institute:

An understanding of the factors controlling the delivery of oxygen and the pickup of CO_2 in the tissues as well as their subsequent movement across the alveolar is of fundamental importance to both the prevention and treatment of heart, lung, and blood disease.

Proposed Course:

The charge effect on glass electrodes will continue to be studied with the further development of the optical pH, PCO_2, and PO_2 catheter electrodes with BEIB. The diffusion OEC apparatus and the OECA TM will be carefully tested as to whether they both give the correct shape of the ODC under all conditions.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01418-05

PERIOD COVERED

October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Fast Responding Oxygen Electrode

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G.G. Vurek	Senior Investigator	NHLBI	LTD

COOPERATING UNITS (if any)

LAB/BRANCH

Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION

NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1/3	1/6	1/6

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work is in progress to develop a composite membrane covered oxygen electrode. The composite electrode should respond rapidly to oxygen partial pressure changes and simultaneously consume little oxygen from the sample.

Methods Employed: A composite membrane made of porous metal or plastic filled with silicone rubber offers a way to control response time independently of effective gas permeability. Parameters of porosity, dimensions and materials control both permeability and response time. The dominant factor for response time is dimension since diffusion down concentration gradients in the driving force for gas movement. Porosity and material composition control permeability. By arranging the geometry so that oxygen passing through the membrane contacts the electrolyte at the cathode, the time required for passage through an electrolyte layer is minimized.

Major Findings: Previous work with materials of porosity varying from 0.15% to more than 1% indicated that bulk porosity has a rather indirect influence on bulk permeability. Subsequent re-evaluation of the data and actual geometry of the membranes suggest the optimum design consists of numerous short parallel holes filled with silicone rubber. The sample end of the holes is covered with a layer of silicone rubber. The other end of the holes is at the electrolyte. By making the holes in silver sheet, the silver can be used as the cathode. Some effort has been expended in obtaining a satisfactory electrolyte resistant bond between the silver and the silicone rubber. Work on this project has been deferred until the last quarter of FY 80 and results are not available for inclusion in this report.

Significance to Biomedical Research and the Program of the Institute:

Conventional oxygen electrodes consume substantial amounts of oxygen per unit area, leading to measurement errors caused by sample stirring or pressure induced dimensional changes. A common approach to the solution of this problem is to use either very small cathodes or thick membranes. These cause substantial time lag problems or electrical measurement problems related to the small currents produced. The composite membrane approach offers a way to overcome these problems and at the same time providing a rugged, easily manufactured electrode.

Proposed Course: A new silicone contact cement has been obtained. The supplier states it is very resistant to water and will provide excellent bonding of silicone membrane to the silver mesh we have been using. The silicone adhesive will fill the holes of the mesh, providing the diffusionbarrier needed as well as holding the outer silicone membrane. Response times and oxygen consumption of test composite structures will be measured.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01421-05 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Development of Electrochemical Methods for Kidney Research and Blood Analysis

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.	:	R. Steele	Physical Scientist	LTD NHLBI
Others	:	J. Handler	Section Chief	KE NHLBI
		J. Johnson	Medical Officer	Walter Reed
		M. Burg	Lab Chief	KE NHLBI
		F. Perkins	Visiting Fellow	KE NHLBI

COOPERATING UNITS (if any)

Laboratory of Kidney and Electrolyte Metabolism, NHLBI

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1	1	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Work on methods and apparatus for the study of kidney and toad bladder epithelial cells grown as sheets on porous membranes continues to yield important results. The central element is the porous bottomed culture device (PBCD) in which the membrane is cemented to polycarbonate rings which provides electrical and chemical isolation between the solutions contacting each side of the membrane. With these PBCD's new knowledge of the development of epithelia is being obtained. For example, the development of vasopressin response in the cells (from African clawed toad kidney) requires that there be free exchange for materials between the cells and the media on the basal side (i.e. the PBCD's must spaced off the bottom of the dish). By this means, vasopressin response has been demonstrated for the first time in these cells.

Work on PBCD's with transparent collagen membranes has been successful and is continuing. Medulary thick ascending limb cells from the rabbit kidney tubule have grown out from a short piece of tubule on 1 mm collagen membranes in about 10 days and produced apical side positive potentials as high as 23 millivolts.

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Objectives:

(1) Develop porous bottomed culture devices with special properties:

- A. Transparent membranes
- B. Membranes thin enough for microscopy and microelectrode work
- C. Small enough for slow growing cells
- D. Very small for growth from one cell
- E. Ultra small for one cell only

(2) Design apparatus for sterile microelectrode work to measure membrane potentials and resistances of individual epithelial cells.

(3) Measure Ca^{++} activity in the media in and below the PBCD's during growth and when there is unexpected change.

(4) Improve the sensitivity of my differential conductometric method for CO_2 measurement.

(5) Measure HCO_3^- binding to albumin.

Methods Employed and Major Findings:

Porous bottomed culture devices, PBCD's have been constructed with 1 and 6 millimeter diameter collagen membranes. These have been useful for observing cells as they grow on these transparent membranes. Cells from the kidney of the African clawed toad (established line A6) were placed on a 6 mm collagen membrane and have been followed for more than 3 months. The PBCD was made with rubber feet which spaced the membrane off the bottom of the dish about 2 mm. At one month, I found that the cells had developed beating cilia. This caused us to consider what other properties these cells may have developed. Thus, a final concentration of 150 milliunits/ml vasopressin on the basal side increased the potential from 16 millivolts to 48 millivolts and was reversible several times. Dr. Perkins has gone on to show that vasopressin response will develope in our regular PBCD's with collagen coated Nucelopore membranes, but only if they are spaced off the bottom. I provided C shaped spacers long ago, but they were considered inconvenient to use. Dr. Johnson has found that a toad bladder cell line, 6C, has twice as great an aldosterone response when the PBCD are spaced off the bottom. Thus, nutrition to be basal side is of great importance in the development of these epithelia.

Many bits of medulary thick ascending limb of rabbit binding tubules have been placed on 1 mm collagen membrane PBCD's. About 50% have grown out to cover the membrane in about 10 days. The majority of these produce potentials ranging from 1 to 23 millivolts, apical side positive. A few preliminary tests which I did indicate that this potential is sensitive to Furosimide and vasopressin. The potential drops to near zero in about 1 month. A carefully controlled set of experiments is being started to follow up on these clues.

The current procedure for making the collagen membrane PBCD's is very time consuming: about 1 hour each. Two layers of collagen are used to cement a collagen membrane to sanded polycarbonate disc with a hole in it. The membrane and each layer are polymizered with amonia and crosslinked with glutaraldehyde. Thorough removal of the glutaraldehyde is critical for cell growth of course. A simpler procedure has just been tried for the 1 mm size and it appears to work. The disc to which the collagen is cemented is cemented to the ring with silicone rubber. It can be pealed or cut away from the ring for special microscopic observation or microelectrode work.

With the help of a summer student Mohit Bhatnagar, the sensitivity of my differential conductometric CO_2 measurement device was increased 10 times. This was done by making the conductivity cells and all other parts of the system 10 times smaller. A better solution pump is now needed.

Significance to Biomedical Research and the Program of the Institute:

The apparatus and methods necessary to grow and study sheets of epithelial cells on membranes is advancing the study of the basic mechanisms of active Na transport. The ability to make the measurements under sterile conditions greatly increases the productivity of this work. Dr. Handler, LKEM, NHLBI, is presenting the physiological significance of this work in detail. The growth of the cells on nutrient and gas permeable membranes results in a degree of development and differentiation which does not occur on the conventional plastic or glass surface. This should allow a study of the development of epithelial that is not possible otherwise.

Proposed Course:

(1) Design apparatus for sterile microelectrode work to measure membrane potentials and resistances of individual cells in a sheet of epithelial cells. Sterility, 5% CO_2 and H_2O vapor will allow long term studies.

(2) Develop very small collagen PBCD's which can be seeded with only 1 cell. The feeder layer often required in cloning can be provided on the other side of the membrane. The feeder cells can grow either in the bottom of the dish holding the PBCD or right on the membrane (planted previously with PBCD upside down).

(3) Measure $Ca^{\#}$ activity in the media in and below the PBCD's during growth and when there is an unexpected change. Micropipettes with 2 to 5 micron tips filled with $Ca^{\#}$ exchange material have proved easy to make.

Publications:

1. Handler, J.S., Steele, R.E., Sahib, M.K., Wade, J.B., Preston, A.S., Lawson, N.L., and Johnson, J.P.: Toad urinary bladder epithelial cells in culture: Maintenance of epithelial structure, sodium transport, and response to hormones. Proceedings of Natl. Acad. Sci. U.S.A., 76, 4151-4155, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01423-04 LTD

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Biophysical Instrumentation for the Study of Protein Dynamics

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	R.L. Berger	Chief, Section on Biophysical Inst	LTD	NHLBI
	B. Balko	Expert Consultant	LTD	NHLBI
	G. Liesgang	Staff Fellow	LTD	NHLBI
	P. Smith	Expert Consultant	LTD	NHLBI
Others:	T. Traylor	Professor of Chemistry	UCSD	
	E. Bucci	Professor of Biochemistry	U. of MD	
	A. Schechter	Chief, Macromolecular Biology Sect.	LCB	A
	G. Hoy	Professor of Physics	Boston U.	
	R. Hendler	Chief, Sect. Membrane Enzymology	LB	NHLBI

COOPERATING UNITS (if any)
Biomedical Engineering and Instrumentation Branch, DR
Department of Chemistry, UCSD

LAB/BRANCH
Laboratory of Technical Development

SECTION
Section on Biophysical Instrumentation

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4	PROFESSIONAL: 2	OTHER: 2

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The Selective Excitation Double Mossbauer (SEDM) apparatus, designed and constructed in this laboratory, has demonstrated that several relaxation processes occur in the model compound tris-pyrrolidine dithiocarbamate Fe (III) (TDC_3). At 4^{o}K, TDC_3 exhibits a spin-flip mechanism of electronic relaxation which requires a change in the electronic spin of $\Delta S_z = \pm 5$. However, at 8^{o}K another relaxation occurs which turns out to be an induced transition between Kramers doublet with electronic spin $S_z = \pm 5/2$ and $S_z = \pm 3/2$ causing a change of $\Delta S_z = \pm 1$. Both processes may be observed at 8^{o}K. Computer programs have been constructed, based on superoperator methods, which generate transmission and SEDM spectra for the general case of an Fe^{57} ion subject to electronic spin fluctuation and lattice dynamics. The picosecond spectrometer has been set up and tested. Considerable work has been done in programming the PAR 1216 Vidicon into a LSI-11.

Objectives:

The objectives of this project are to develop new instrumentation, data handling techniques, mathematical methods, and models of the dynamics of energy exchange, conformational change and other dynamic phenomena involved in the reaction of proteins with their coenzymes and substrates. In particular to develop methods and instruments to study the local environment of the iron ion in hemoglobin, the cytochromes and other iron proteins under various conditions, such as during oxygen binding to the hemoglobin tetramer and the α and β chains individually, and the electron transfer in cytochromes.

Model reactions will be studied with especially prepared hematin compounds using replacement and pyrrole ring substitutes as well as naturally occurring variants in an attempt to understand the fundamental relations occurring in the iron protein reactions.

Methods Employed:

The methods employed are those of Mossbauer effect spectroscopy in the transmission, scattering, and selective excitation double Mossbauer (SEDM) modes, and picosecond laser spectroscopy. These involve cryogenic techniques which allow a range of sample temperatures of .05°K to 330°K.

Major Findings:

I. Selective Excitation Double Mossbauer (SEDM)

The SEDM apparatus has been designed, assembled and tested for the study of iron containing biological compounds. The experiments performed on the polypeptide, ferrichrome A (FA), were designed to be a test of the general theory of the SEDM lineshape. However, we found that even in this apparently simple and well studied text book model system, the mechanism of electronic relaxation between energy levels, which determines the dynamic environment of the iron, is actually different from the one described by the presently accepted theory. Thus the sensitive,selective,and discriminating SEDM instrument has already revealed new information about a biological system.

In preparation for the application of this technique to the investigation of more complex biological systems the SEDM technique had to be tested and the instrument calibrated on a well defined system. Tris-pyrrolidine dithiocarbamate F^{e} (III) (TDC$_3$) is such a system. It exhibits a Mossbauer lineshape which can easily be interpreted in terms of a simple model of electronic relaxation between energy levels. This compound was used to check the experimental technique and establish the validity of the general SEDM lineshape expressions and their applicability to the investigation of heme proteins and other iron coupled energy systems.

Unlike ferrichrome A which we investigated earlier, SEDM experiments on TDC$_3$ at 5.4 K confirmed the existance of a "spin-flip" mechanism of electronic relaxation, in agreement with ESR and Mossbauer transmission measurements.

However, at 8K another mechanism which had not been observed earlier. This mechanism induces transmissions between Kramers doublets with electronic spin $S_z = \pm 5/2$ and $S_z = \pm 3/2$ causing a change of one unit of angular momentum. At 8K we were actually able to observe directly energy exchange due to both of these mechanisms acting simultaneously.

In support of the experimental work we have developed computer programs SPIN52, RELAX 1 and HB1 which are based on superoperator mathematical techniques. These programs generate theoretical Mossbauer transmission and SEDM spectra for a general case of an iron 57 ion subject to electronic spin fluctuations and lattice dynamics. Using these programs we analyze the experimental data, interpret results, and obtain the molecular and electronic dynamics for the iron containing compounds.

Using SPIN52 to analyze the transmission Mossbauer effect spectra of ferrichrome A we found that not only was the relaxation mechanism incorrectly determined by early investigators but also the parameters D, E and the tensor A which describe the interaction between the ^{57}Fe nucleus the electrons and the molecular environment, D gives the electronic level splittings, E the amount of level mixing and A hyperfine interaction. Again this shows that SEDM and the correct general theory must be used to interpret ME spectra of biological molecules. Otherwise even in the simple compounds like FA and TDC_3 gross misinterpretations can result.

Our SEDM results are important because a) they prove the superiority of SEDM, in terms of greater selectivity, discrimination and resolution, over other techniques in studying electron dynamics and energy transfer in molecules. Two model "textbook" systems (FA and TDC_3) which were thought originally to have the same relaxation mechanism were discovered by SEDM to have two completely different mechanisms. Also two different mechanisms were shown to exist in TDC_3.
b) Since most biological compounds show even more complex Mossbauer spectra it is imperative to study them with SEDM, both to decouple the various effects and separate lines belonging to different sites, charge states or spin states as well as to study the energy transfer through the measurement of electronic relaxation. This information is important to the ultimate understanding of the biological function of the molecule.

II. Transmission Mossbauer Effect (ME) Spectrometer

We have expanded the capability of our transmission ME spectrometer by incorporating into the apparatus a Lake Shore Cryotronics, Inc. cryogenic furnace. This device is capable of cooling samples in 2 min. to 150 K from a cold start thus allowing a quick sample change without thawing the sample. This quick freezing process is important in preventing protein denaturation. This system is connected directly to the reservoir of the cryogenic liquid thus obviating time consuming and costly periodic transfer of He or N_2. The transmission system has been used to investigate the application of Mossbauer spectroscopy to several biological problems.

A. Model Compounds

Model compounds of hemoglobins provide an obvious way of investigating dioxygen binding. By varying the structures and environment of the heme iron in these compounds the specific properties of the tense and relaxed states of the hemoglobin molecule can be investigated. In the application of the Mossbauer techniques to this problem we found a direct relationship between the separation of the lines in a Mossbauer spectrum and the strain induced on the proximal base in model compounds of myoglobin prepared by Professor Teddy Traylor of UCSD. This correlation between the strain on the proximal base, pulling it away from the heme plane and the electronic spin and valence state of the iron ion will permit us to investigate in the most detailed way the molecular-mechanical model of the heme involvement in hemoglobin cooperativity.

B. Sickle Cell Hemoglobin - Onset of Gelation

We have investigated novel approaches to the solution of biological problems and have produced a new technique for measuring microviscosity of solutions. In cooperation with Dr. Alan Schechter of NIADD we are adapting it to the study of gleation of HbS. The technique consits of injecting coated 100 angstrom particles of ferrite into the solution and observing changes in the Mossbauer lineshape due to the Brownian movement of the particles.

Such studies will provide a new and important probe of the onset of gelation, because the particle dynamics are extremely sensitive to microviscosity. Also the size of the particles can be varied thus varying the size of the volume probed, whereas, other microviscosity techniques are limited to a single characteristic probe volume. Our technique can probe volumes from hundreds to thousands of cubic anstroms.

C. High Potential Iron Sulfur Proteins

ME transmission has also been applied to the study of high potential (HiPip) iron sufur proteins which show a particularly high affinity for multiple electron transfer. These proteins were prepared enriched in ^{57}Fe by growing the bacteria in a rich ^{57}Fe environment. Preliminary experiments show that we can observe both the reduced and oxidized forms. The effect of a weak (1150 gauss) magnetic field enhances the resolution and therefore stronger (up to 10 tesla) fields will be used to bring out details of the spectra.

D. Sample Enrichment in ^{57}Fe

Because the signal to noise in ME experiments is directly proportional to the amount of ^{57}Fe in the sample, various approaches to enrich the samples in ^{57}Fe are being investigated. In particular we are preparing

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enriched hemoglobin by introducing ^{57}Fe into reticulocytes in culture. Another attempt is to chemically replace the iron. This is a most promising technique because it may provide a means of selectively enriching the α and β sites and make it possible to study these sites independently in a reconstructed tetramer.

III Picosecond Laser System

Vidicon-Computer Interface

A Digital Equipment Corporation (DEC) MINC LSI-11 based computer was interfaced with a Princeton Applied Research (PAR) vidicon. This interface enables access to three dimensional spectroscopic information, in the picosecond time region, by dispersing the output of a monochromator (Jarrell-Ash 82-00 series 0.5 meter spectrometer) on the face of the vidicon. Time is represented on a perpendicular axis on the vidicon by stepped time delays (ΔT = 0, 5, 10, 15, etc. picoseconds) of the interogating laser pulse. Intensity, of course, is read as the amount of charge remaining on the vidicon photodiode array after exposure. In order to access this 3-D information the vidicon surface must be read as discrete channels of wavelength, for each given time delay (a track). The vidicon is preprogrammed to achieve this by the operator, via the MINC computer system. Upon the vidicon's receipt of a run command, the preprogrammed scan is activated and the digitized value of intensity for each wavelength/time pair is presented to a digital port. This digital port is accessed, via an assembly (MACRO) language routine and the 16 bits of data (per each channel) is stored in RAM memory. Since one complete scan of the vidicon surface reads only 70% of the actual vidicon signal, four successive scans are required in order to read more than 99% of the intensity value. This successive scanning requires "real-time" integration of each new scan with the previous scan. This is done via an assembly language routine. Once the information has been accessed from the vidicon the operator has the option either to repeat the data or to permanently save it on disk for future analysis.

The program referred to above has been designed to allow an interactive approach to entering the parameters required for desired scan pattern. In addition to allowing a simple, straightforward method of entering the parameters, the program has features to check the various parameters entered to ensure that they fall within legal bounds for scanning the vidicon surface. This feature is of importance to prevent a scan pattern of the readout electron beam damaging the vidicon target surface; it is still possible to scan small areas and to be in legal parameter bounds, in which case a warning message is displayed which must be answered affirmatively to allow the program to proceed. The program in its present form allows automatic control of the vidicon and laser during an experiment; the charging, firing, and timing of the laser pulse with the Vidicon's reading sequence are all under program control.

At present, the size of the scanned array is limited to 16,000 pixels which can be any valid combination of scanned channel and track parameters; e.g. 100 tracks (5 elements high), 160 channels (4 integrations wide) scans 480 x 500 of the available 500 x 500 pixels. The limit of the number of pixels is determined by the memory made available by the MINC Fortran compiler. This limitation can be overcome by writing more of the program in assembly language routines.

The vidicon has been tested for linearity in terms of 1) the number of scans required to achieve the maximum signal-to-noise ratio at various signal levels, 2) the effect of the number of prep frames 3) the effect of varying the time spent recording each channel. In summary, a dark count of 3 per pixel is obtained, which rose to a maximum of 132 per pixel at high light levels. At the higher light levels, 2-3 scans reads the signal to >90% with more scans increasing the noise at the expense of signal. In addition, if a multi-track scan is performed, charge leakage occurs at the periphery of the scanned area; PAR warns about this problem in one direction and allows programming of the vidicon accordingly. A similar problem exists in the perpendicular direction and an area equivalent to the first 10 pixels has to be discarded.

The information presented on the vidicon target from a pulsed source, lasts only one second or less at room temperature owing to charge leakage. For this reason, the timing of the laser pulse with data acquisition becomes essential. A hardware interface has been designed, constructed, and implemented which handles the charging of the laser, firing the laser, and informing the assembly program when to start accessing data from the vidicon surface.

The DEC LSI-11 computer is a laboratory minicomputer which means the data obtained on the MINC must be transferred to a larger mainframe computer system for perfroming detailed numberical analysis of the data. With the aid of software written by DCRT, a communicative link was established between DEC LSI-11 MINC and the NIH Dec-system-10. The transfer of information is carried out over a Bell Telephone modem operating at 1200 band.

It should also be noted that the Vidicon-MINC interface is not restricted to accessing only picosecond spectroscopic data. It has been designed so as to operate as a general 3-D spectroscopic imaging device. As an example, the vidicon will be used to develop a stopped flow technqiue emplying Raman scattering detection.

The output of the neodymium glass picosecond laser system has been optimized for oscillator-amplifier firing delay. A single pulse energy of 100 mJ is obtained from the amplifier and has been measured using a Laser Precision, model RS7100 thermopile calorimetric detector. The amplified pulse at 1.06μ has been frequency doubled to 530 nm using a

Type I KDP crystal angle-tuned to the phase-matching angle. Synchronous picosecond continua have been produced by focussing down either the 530 nm light or 1.06μ light into a variety of solvents such as water, ethanol, and carbon tetrachloride. Each solvent produces a white light continuum with intenser bands at various spectral regions which can be exploited for monitoring purposes. The purpose of this continuum is to provide a method of studying the reactions to be initiated by the 530 nm pulse. The duration of the continuum is the same as the 530 nm or 1.06μ pulse. To provide a sequence of monitor media. In air, light travels at 3mm/ps; by splitting the continuum into segments with each segment taking a longer path than the preceding segment, each segment can be made to arrive at the sample at a different time. A quartz echelle (working on differential time of flight in two media-quartz and air) has been obtained to provide temperal dispersion to 200 ps and a reflection temporal disperser has been manufactured in our workshop to accommodate longer time events to 2 nanoseconds.

A pressure vessel has been designed to provide 100 atm of pressure. This is being constructed in our workshop. The design allows the whole unit to be taken down to liquid helium temperatures.

Proposed Future Research

The picosecond spectrometer construction and testing will continue. A high pressure cryogenic cell will be constructed for inclusion in the system as well as direct coupling to a PDP-11 computer. The SEDM and transmission spectrometer development will continue with construction of a helium dilution cryostat for 50 milikelvin, or lower, operation. SEDM investigation of enriched hemin and model compounds as well as hemoglobin chains and the whole molecule will continue. The application of ferrifluids to the study of the mechanism and the onset of gelation will continue. The development of the appropriate mathematical theory and computer programs to describe the SEDM and transmission lineshapes for the most general conditions of hyperfine fields and relaxation rates will continue with particular attention being paid to the ferrous state since the ferric state programs are now operational.

Significance to Biomedical Research and the Program of the Institute

The detailed dynamics of molecular interactions between parts of the protein molecule during ligand reaction is needed for the understanding of the mechanism of all protein reactions. Thus the Mossbauer and picosecond research instrumentation hold the promise of greatly extending our understanding of the mechanism by which the hemeproteins, such as hemoglobin and the cytochromes react. This is of fundamental importance for the understanding of and the prevention and treatment of heart, lung, and blood diseases.

Publications

1. B. Balko, G. R. Hoy: Selective Excitation Double Mossbauer Spectroscopy in Advanced Techniques in Mossbauer Spectroscopy - Application to Physics, Chemistry and Biology, edited by B. V. Thosar and P. K.Jyengar, Elsevior Scientific Publishing Co, Amsterdam, in press.

2. B. Balko: Application of Mossbauer Spectroscopy to Hemoglobin Studies in Hemoglobins, a Volume of Methods in Enzymology, edited by L. Rossi-Bernardi, E. Antonini and E. Chiancone, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01428-03 LTD

PERIOD COVERED
October 1, 1979, through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Application of the New Horizontal Flow-Through Coil Planet Centrifuge for Separations of Synthetic Peptides

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoichiro Ito	Medical Officer	NHLBI	LTD
	Gerald Putterman	Medical Officer	NCI	Fred. Cancer Center

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI ,NIH, Bethesda, Maryland 20205

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1.0	1.0	

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Countercurrent chromatographic method previously reported (Z01 HL 01428-02) has been applied to preparative separation and purification of two synthetic polypeptides with 5 and 15 amino acids. The crude samples were purified with a two-phase solvent system composed of normal butanol, acetic acid, and water at a 4:1:5 volume ratio with a satisfactory level of recovery. The present method was proved to be well applicable to peptide samples which had poor solubility in the solvents commonly used in the conventional liquid chromatography.

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Project Description:

Objectives: Separation and purification of crude synthetic peptides by means of countercurrent chromatography with the new horizontal flow-through coil planet centrifuge.

Methods Employed and Major Findings:

a) Apparatus: The design and the function of the new horizontal flow-through coil planet centrifuge were given earlier (Z01 HL 01428-02). The apparatus holds a pair of coiled separation columns one suitable for analytical-scale work and the other, for preparative-scale work. In the present studies, the preparative column was exclusively employed to separate a relatively large amount of samples. The column consisted of a 50 m long, 0.26 cm i.d. PTFE tube with 1000 helical turns (1.25 cm helical diameter) and a total capacity of about 270 ml. A Chromatronix metering pump was used to pump the solvent and an LKB Uvicord III for continuous monitoring of the eluate at 280 nm while an LKB fraction collector was used to collect fractions for further analysis.

b) Experimental: The two-phase solvent system was prepared by mixing normal butanol, acetic acid and water at a volume ratio of 4:1:5 in a separatory funnel at the room temperature. Two peptide samples synthesized by a modified Merrifield solid-phase method were chosen for the present study. One peptide (Peptide I) had the sequence ser-ser-ile-ile-arg and the other (Peptide II), tyr-ala-ala-nle-ala-ala-met-arg-asp-val-cal-leu-phe-glu-lys. Each crude peptide sample was dissolved in either upper or lower phase of the above solvent system.

For each separation a column was first filled with the stationary phase (either upper or lower phase). The sample solution was then injected through the sample port into the column which was rotated at the desired revolutional speed. Then the mobile phase was pumped into the column at a desired rate. The eluates were continuously monitored with an LKB Uvicord III at 280 nm and fractionated with an LKB fraction collector.

The obtained fractions were vortexed whereupon aliquots were removed and spotted on MN cellulose 300 plates (Brinkmann, Westbury, N.Y.). TLC was performed with the solvent system of normal butanol, acetic acid and water (4:1:5 v/v). Peptides were detected by means of a collidine-containing ninhydrin reagent. A fluorescence profile of the fractions was obtained with an SPF-500 spectrofluorometer (American Instrument Co., Silver Spring, MD.) in the single-beam mode. The wavelength for excitation was 280 nm, and fluorescence emission was determined at 308 nm using a 5-nm bandpass for both monochromators. Amino acid compositions and yields of the synthetic peptides were determined with a Durrum Instruments (Sunnyvale, Calif.) D-500 amino acid analyzer equipped with a fluorescence detector. Amino acids were made fluorescent by reaction with o-phthalaldehyde. In order to compare the results obtained by the present method with that in the conventional technique, Bio-Gel P-2 chromatography was applied to Peptide I. Crude peptide (100 mg) was dissolved in 2 ml of 0.1 M acetic acid and applied to a 60 x 1.5 cm column of Bio-Gel P-2 (Bio-Rad Labs., Richmond, Calif.). Elution

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was performed with 0.1 M acetic acid.

c) Results: Peptide I (100 mg crude material) was purified by eluting the column with the lower aqueous phase at a flow rate of 16 ml/hr while the apparatus was spun at 500 rpm. Twenty fractions (each 2 ml) were collected. The amino acid analysis clearly revealed that tubes 11-16 contained the desired peptide; tubes 3-6 contained serine plus traces of other contaminants; tubes 7-10 contained both the desired peptide and serine. The yield of pure peptide was 65%, but this should be improved by rerunning tubes which contain both the peptide and serine contaminant. In four runs, the pure peptide was found 24 ± 2 ml after the solvent front. The separation of peptide I with the Bio-Gel P-2 chromatography revealed that the fractions which had the desired peptide also contained white material which remained at the origin and did not stain with peptide reagents. Either a much larger column or repetitive runs would have to be used to obtain good yields of uncontaminated peptide.

The peptide II (100 mg crude material) was purified as for the peptide I except that the non-polar upper phase was used as the mobile phase. Although monitoring of ultraviolet absorbance was used to locate tubes of interest, improved sensitivity was obtained by monitoring with fluorescence, yield of peptide, amino acid composition and behavior on TLC. The results indicated that the run could be divided into three main areas of interest. The high levels of fluorescence for the contents of tubes 5-11 and their lack of reaction with ninhydrin when run on TLC suggest that these tubes contained blocked peptides and aromatic by-products of deplocking. The hypothesis is further supported by the realization that, by using the non-polar phase as the mobile phase, the least polar products would appear in the early tubes. Amino acid analysis also suggests that this fraction contained abbreviated sequences. Tubes 38-49 contained the pure peptide as indicated by amino acid analysis and uniform TLC behavior. Tubes 50-66 contained peptide contaminated with tyrosine from insufficient washing of the resin following the last coupling. The recovery figure of the peptide was then raised to 53% which considered to be satisfactory level, since the peptide was less soluble in the aqueous solvent typically used for ion-exchange chromatography and gel filtration.

Significance to Biomedical Research and the Program of the Institute:

The new horizontal flow-through coil planet centrifuge can perform efficient preparative separations of various biological samples. Among a variety of applications, separation and purification of natural and synthetic peptides may be the most useful. With synthetic peptides, a planet centrifuge run appears to free the desired peptides of salts, resin by-products, and contaminating peptides. Even with a peptide possessing low solubility, yields can be improved by recovering the column contents without the complications of the peptide precipitating on a support medium. The present method will be extremely useful in separation of purification of various peptide hormones in the biochemical and biomedical researches.

Proposed Course: Application of the present method to separation of various peptide hormones and other samples.

Publications:

1. Ito, Y. Countercurrent Chromatography with a New Horizontal Flow-Through Coil Planet Centrifuge, Anal. Biochem. 100, 271-281 (1979).

2. Ito, Y. A New Horizontal Flow-Through Coil Planet Centrifuge for Countercurrent Chromatography: I. Principle of Design and Analysis of Acceleration J. Chromatography 188, 33 (1980).

3. Ito, Y. A New Horizontal Flow-Through Coil Planet Centrifuge for Countercurrent Chromatography: II. The Apparatus and its Partition Capabilities. J. Chromatography 188, 43 (1980).

4. Ito, Y., and Putterman, G.J. A New Horizontal Flow-Through Coil Planet Centrifuge for Countercurrent Chromatography. III. Separation and Purification of Dinitrophenyl (DNP) Amino Acids and Peptides. J. Chromatography 193, 53-60 (1980).

5. Putterman, G.J., Perini, F., White, E.L., and Ito, Y. Purification of Synthetic Peptides by Means of Countercurrent Chromatography with the Horizontal Flow-Through Coil Planet Centrifuge. Peptides: Structure and Biological Function, Proceedings of 6th American Peptide Symposium. Ed. Gross, E. and Meienhofer, J., Pierce Chemical Co., page 113, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01434-02 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
Microcolorimetric Measurement of Magnesium Ions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	G. G. Vurek	Senior Investigator	LTD	NHLBI

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 1	PROFESSIONAL: 2/3	OTHER: 1/3

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☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Measurement of picomole amounts of magnesium ion by colorimetry can be accomplished with the aid of a new microcolorimeter. The working volume of the colorimeter is less than 300 nL. Using continuous-flow analysis, not only magnesium but also calcium and inorganic phosphate can be measured with femtomole sensitivity.

PHS-6040
(Rev. 10-76)

Objectives: The objective of this project is to explore new designs for microcolorimeters and demonstrate their performance by measurement of picomole amounts of magnesium and other ions and substances.

Methods Employed: A cuvet has been constructed of quartz tubing with two right-angle bends one cm apart; the tubing inside diameter is 0.17 mm. Using micro quartz working techniques, thin windows are blown in the bend regions. These windows pass light from a source through the sample axially, and out of the tube to a photosensor. This provides a smooth fluid path which does not easily trap bubbles. Moreover, the quartz material passes not only visible but ultraviolet wavelengths, making the colorimeter useful for measurements over a wide range of wavelengths. Stray light passing through the walls of the capillary are eliminated by coating a portion of the outer surface with a carbon black-immersion oil mixture.

As currently used, the curvet is connected to a continuous flow or "flow-injection-analysis" system. A steady stream of reagent passes from a reservoir through a sample injection port to the cuvet and out to a syringe pump. Flow rates are adjusted to suit analytic requirements but typically are a few microliters per minute. Nanoliter volume of samples are injected through a mercury drop which seals the injection port.

A light emitting diode and silicon photo sensor are used as source and detector for the system sample. Filters are used to eliminate room light effects.

Major Findings:

The new one-piece quartz cuvet offers considerable advantages over earlier designs. It is easy to fabricate, has a smooth flow path of uniform material, and passes not only visible but ultraviolet wavelengths. Although fiber optic elements can be sealed into the path, these add hydraulic discontinuities which trap bubbles. Adequate optical properties are obtained by blowing thin windows at corners of the cuvet. The working volume of the one cm path cell is 250 nL.

The cuvet is arranged to be part of a continuous flow or "flow injection analysis" system. A stream of reagent passes continuously by an injection part through the cuvet to a syringe pump. The injection port is a Tee; a drop of mercury seals the junction. Nanoliter volume pipets can be used to inject samples through the mercury with the reagent stream. The injection port is at a low pressure point of system to prevent the mercury drop from lifting out of the seal. A syringe pump draws reagent through the system. Typical flow rates are about 2.5 μL/min. The present instrument uses a light-emitting diode (LED) (655 nm peak) as a source and a silicon photodiode as a sensor. A set of glass filters in front of the photodiode restricts the light reaching it to red wavelengths; this minimizes interference from stray light. The LED output is modulated at 162 Hz; a lock-in amplifier selectively amplifies the signal for recording and integration. We use a baseline correcting integrator to quantitate the sample signal. Absorbance linearity is maintained up to 1.5 absorbance units.

We have demonstrated the performance of the system by applying it to the determination of picomoles amounts of magnesium. By using a commercial reagent kit for clinical magnesium measurements, we can demonstrate the high sensitivity of the apparatus with well characterized reagents. This kit uses a reagent which complexes calcium and other interfering ions and permits magnesium ions to bind to a metachromatic dye. The dye (common name: calmagite) absorbs strongly at 655 nm and the additions of magnesium increases the transmittance. On a macro scale, the absorbance decrease is linearly proportional to the magnesium concentration. However, the injection of small (10 nL) volumes into the flowing reagent stream produces only small (a few percent) changes in the transmittance and these changes are linearly related to the amount of magnesium injected. Initial tests indicate a sensitivity of 0.5 picomole, which is comparable to present microfluorometric techniques and the results obtained with the helium glow photometer.

In addition to magnesium, there are reagent kits for calcium and inorganic phosphate which use cherogenic reagents with useful absorbance changes at 655 nm. Preliminary tests show the new microcolorimeter suitable for these materials as well.

Significance to Biomedical Research and the Program of the Institute:

This new colorimeter offers a simple rapid method for analyzing a wide variety of materials at the picomole level. In addition it can be used as a chromatographic column monitor for very small amounts of sample and microbore chromatographs.

Proposed Course:

We will complete the demonstration of the performance of the apparatus for the measurement of magnesium, calcium, and phosphate. In addition, some consideration will be given to the construction of a variable wavelength source to increase the flexibility of the system.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01436-01 LTD

PERIOD COVERED
October 1, 1979, to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Non-synchronous flow-through coil planet centrifuge without rotating seals

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoichiro Ito	Medical Officer	NHLBI	LTD
	Peter Carmeci	Electrical Engineer	NHLBI	LTD
	Stephen Leighton	Mechanical Engineer	BEIB	DRS

COOPERATING UNITS (if any)

Biomedical Engineering and Instrumentation Branch, Division of Research Sciences

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.0	1.0	1.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A new non-synchronous flow-through coil planet centrifuge allows continuous elution through the coiled separation column without the use of rotating seals. Rates of rotation and revolution of the coiled column are independently adjustable to meet the requirements for the separation of cell particles. Thus the slow rotation of the column under a high revolutional speed produces a chromatographic separation of cells according to their size and density. The capability of the present apparatus was demonstrated by the separation of human and sheep erythrocytes with a buffered isotonic saline solution. The method has been successfully applied to separations of T and B lymphocytes, malaria parasites (gametocytes), and liver cells, with physiological culture media.

PHS-6040
(Rev. 10-76)

Project Description:

Objectives: Development of non-synchronous flow-through coil planet centrifuge without rotating seals and application of the apparatus to separation of cells with a physiological solution.

Methods Employed and Major Findings:

a) Apparatus: The apparatus is equipped with two motors, the first motor providing the revolution of the column and the second introducing the rotation of the column about its own axis. The first motor drives a pair of identical planetary gear sets (A and B) rigidly linked together each engaged to an identical internal ring gear (A and B respectively). Each individual planetary gear is coaxially connected to an idler gear which in turn interlocks to the respective central sun gear (A and B). The tooth ratio between these three types of gears is selected as 1 for idler gear: 2 for planetary gear: 3 for sun gear so that the sun gear rotates twice the speed of the planetary gear set when the internal ring gears are kept stationary with respect to the earth. Sun gear A is directly connected to a rotary frame which is coaxially mounted between the two planetary gear sets and it holds a pair of column holders in the symmetrical positions at 12 cm away from the central axis of the apparatus. One of the holders is equipped with a toothed gear which engages to an identical gear directly connected to the sun gear B through a toothed belt. The internal ring gear A is driven by the second motor while the internal ring gear B is kept stationary with respect to the earth at all times. With this gear arrangement, the rotational rate of the column holder can be conveniently adjusted by choosing the rate of the second motor. Consequently, the rotation/revolution ratio of the holder becomes freely adjustable. The system also eliminates the use of the rotating seals and permits continuous elution through the column without complications such as leakage, contamination and clogging the channels at the site of the seals. The revolutional rate of the apparatus can be adjusted up to 1200 rpm which provides a centrifugal force field of 164 x g on the coiled column.

The separation column was prepared from a piece of 0.55 mm i.d. PTFE tubing by winding it onto six units of stainless steel tubes (0.68 cm o.d. and 20 cm long) in a series. The entire column consisted of 1200 helical turns with a total capacity of about 6 ml. The column with a different diameter of the tubing can also be used. The column was symmetrically arranged the holder shaft and tightly screwed to the column holder through a hole made at each end of the column units. The counterweight is applied to the other dummy holder to balance the apparatus.

b) Methods and Materials: Isotonic buffered saline solution (pH 7.4) was prepared by dissolving NaCl, 90 g; Na_2HPO_4, 13.65 g; and $NaH_2PO_4 \cdot H_2O$, 2.15 g in one liter of distilled water and diluting 85 ml of this stock solution with distilled water to bring the final volume to one liter. In order to prevent the adhesion of cells to the tube wall, bovine serum albumin (BSA) 35% solution was added to the above solution to make the concentration of BSA at about 0.5%.

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The human erythrocyte suspension was prepared from EDTA-treated, fresh adult blood by washing with the buffered saline solution three times, repeating centrifugation and decanting the supernatant. One part of the loosely packed cells thus obtained was finally mixed with 4 parts of the same solution. The sheep erythrocyte suspension was similarly prepared from ACD-treated blood stored at $4^{o}C$. Equal volumes of human and sheep erythrocyte suspensions were combined and 0.2 ml of this mixture was used for each separation.

The separation was performed as follows: The coiled column was first filled with the buffered saline solution followed by injection of the sample through the sample port. Then the column was eluted with the buffered saline solution at a flow rate of 5 ml/hr with a pump while the apparatus was spun at 800 rpm combined with 10 rpm column rotation. The eluate was collected into test tubes at 10 minute intervals. Fractionated samples were analyzed microscopically with a blood counting chamber to obtain the total cell population together with the percentages of human and sheep erythrocytes. Original samples and some fractions were also analyzed with a Coulter size distribution analyzer.

c) Results: The sample was separated into two peaks. The microscopic examination revealed that the first peak almost entirely consisted of the sheep cells and the second peak, the human cells. The overall picture of the elution curve of the cells closely resembled the size distribution curve of the original sample mixture obtained by the Coulter analyzer. Also, the analysis of the fractions by the Coulter analyzer showed that the cell size increases with the fraction number. These findings strongly indicate that the present method separates cell particles according to their size difference. Microscopically the cells were well preserved and no evidence of hemolysis was observed during separation. The recent method has also been applied to separations of T and B lymphocytes, malaria parasites (gametocytes), and rat liver cells. T and B lymphocytes from the rat spleen were almost completely separated when the cells were labelled with fluorescone-Ig, while no separation was observed on non-labelled samples. Human malaria gametocytes were enriched over 80% from 5% in the original sample suspension. In the separation of rat liver cells damaged cells were almost completely elated out in a sharp peak followed by a broad peak of intact cells which were apparently spearated by their size of aggregates originally present in the sample suspension. In order to achieve successful separation, an improved sample prepatation to obtain homogenously dispersed cell suspension is required.

Significance to Biomedical Research and the Program of the Institute:

Most conventional cell separation techniques utilize non-physiological media which may alter the physiological function of cells. In the present methods cells are only exposed to a physiological solution for a relatively short period of time so that the normal cell functions may be well preserved. In addition, the present apparatus uses a rotating-seal-free flow-through system to eliminate various complications such as leakage, contamination and clogging of the channels at the site of the seal. Therefore, the present method will become a useful tool for separation of vulnerable cells in biochemical and biomedical researches.

Proposed Course:

1. Refinement of the apparatus to eliminate unnecessary noise and vibration.

2. Application of the method to various cell samples.

3. Application of the method to partition of cells and macromolecules with polymer phase systems.

4. New application of the apparatus.

Publication:

Ito, Y., Carmeci, P., Bhatnagar, R., Leighton, S. and Seldon, R.: The Non-Synchronous Flow-Through Coil Planet Centrifuge without Rotating Seals Applied to Cell Separation. Separation Science and Technology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01437-01 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)
The Toroidal Coil Planet Centrifuge without Rotating Seals for Performing Countercurrent Chromatography

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoichiro Ito	Medical Officer	NHLBI	LTD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

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INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A simple tabletop model of the versatile countercurrent chromatographic system is introduced. The apparatus compactly holds a long coiled column around a drum-shaped holder. The holder undergoes a synchronous planetary motion in such a way that it revolves around the central axis of the apparatus and simultaneously rotates about its own axis at the same angular velocity in the same direction. The acceleration produced by the synchronous planetary motion of the holder enables stable retention of the stationary phase in each helical turn of the coiled column while the mobile phase is continuously eluted through the column. The capability of the method was demonstrated on separations of DNP amino acids and oligopeptides using typical two-phase solvent systems. The present method enables universal application of two-phase solvent systems to separate a variety of biological samples without complications arising from the use of solid supports.

Project Description: Development of the toroidal coil planet centrifuge for performing countercurrent chromatography.

Methods Employed & Major Findings:

a) Apparatus: The rotary frame of the apparatus consists of a pair of circular aluminum plates rigidly bridged together with multiple links and is driven by a motor (ElectroCraft) around the stationary pipe mounted on the central axis of the centrifuge. The frame holds a pair of cylindrical coil holders, one 15 cm in diameter and the other 10 cm in diameter, in symmetrical positions 10 cm away from the central axis of the centrifuge. Each coil holder is equipped with a plastic gear which is engaged to an identical stationary pipe. This gear arrangement produces the desired planetary motion to each coil holder, i.e., revolution around the central axis of the apparatus and rotation about its own axis at the same angular velocity in the same directions. In order to reinforce the stability of the centrifuge, the free end of the rotary frame is coaxially connected to a short coupling pipe which is in turn supported by a stationary member through a ball bearing.

The separation column is prepared by winding PTFE tubing (Zeus Industrial Products, Raritan, NJ) onto a flexible core which is again coiled around one of the coil holders to make a coiled helix configuration. A counterweight is applied to the other coil holder to balance the centrifuge. The flow tubes from the column are first passed through the center of the coil holder shaft and then led into the opening of the central stationary pipe through a side hole made on the short coupling pipe. These flow tubes are lubricated with silicone grease and protected with a piece of plastic tubing at each supported portion to prevent mechanical damage. The planetary motion produced by the gears permits the flow tubes to simply roll around themselves without twisting. The revolutional speed of the test system is continuously adjustable up to 1000 rpm.

b) Analysis of Acceleration Field: Analysis of acceleration field at various locations on the rotating holder was made by the aid of a coordinate system. The results indicate that the centrifugal force field greatly varies according to the location of the point on the holder which is conveniently expressed as beta, the ratio between the radii of rotation and revolution of the point. When beta is greater than 0.25, the centrifugal force vectors always directed outwardly from the holder. In addition, the centrifugal force vector undulates in its relative magnitude and direction during one revolutional cycle of the holder. As the beta value increases, the magnitude of the relative centrifugal force vector becomes greater while the amplitude of the angular oscillation is reduced. This distribution pattern of the force vector provides a great advantage when the scheme is used for performing countercurrent chromatography. Under the above centrifugal force field the two phases in the coiled column are separated in such a way that the heavier phase occupies the outer half and lighter phase, the inner half of each coil unit. Angular undulation of the force vector efficiently mixes the interface and the two phases in each turn of the coil to reduce the mass transfer resistance.

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Consequently, solutes introduced locally at the inlet of the column are subjected to an efficient partition process and separated according to their relative partition coefficients as in liquid partition chromatography but in the absence of solid supports.

c) Studies on Partition Capabilities of the Apparatus: The performance of the present apparatus relies upon retention of the stationary phase and efficiency of solute partitioning between the two solvent phases in the column. In order to determine the optimum operational conditions, a series of experiments were performed to study the degree of stationary phase retention and partition efficiency in short coiled columns under various revolutional speeds and flow rates.

Two types of coiled columns were prepared each from a 7 m long, 0.55 mm i.d. PTFE tube winding it onto a flexible nylon core of different diameters. One column has a core diameter of 5 mm and the other column, 1.5 mm. In both columns, the total capacity measured was approximately 2 ml. These columns were mounted on the column holder having beta value of 0.75. Two typical phase systems were selected, $CHCl_3/CH_3COOH/0.1N\ HCl$ (2:2:1 volume ratio) for partition of DNP amino acids, and $n\text{-}BuOH/CH_3COOH/H_2O$ (4:1:5 volume ratio) for partition of oligopeptides. For each phase system a pair of samples with suitable partition coefficients were selected: N-DNP-L-glutamic acid and N-DNP-L-alanine for the chloroform phase system; L-valyl-L-tyrosine and L-tryptophyl-L-tyrosine for the normal butanol phase system.

The overall results indicate that the satisfactory level of retention is obtained over a wide range of revolutional speed and flow rates for both solvent systems. The 5 mm core column gives a slightly higher level of retention than the 1.5 mm core column. Because of the solvent-wall interaction in the narrow-bore tube, the nonaqueous phase having an affinity to the PTFE tube gives substantially higher levels of retention than the aqueous phase under a given set of operational conditions. The retention levels of the normal butanol phase system is generally lower than those of chloroform phase systems due to its higher viscosity and the smaller difference in density between the two phases. Satisfactory retention levels obtained from these typical phase systems with contrasting physical properties suggest that the present method permits universal application of the two-phase solvent systems.

Overall results in partition efficiency studies indicate that the 1.5 mm core column yields higher peak resolution than the 5 mm core column. Although good peak resolutions are given at 6 ml/hr flow rate in short periods of time, the highest partition efficiency is obtained at 2.4 ml/hr flow rate under revolutional speeds of 600 to 1000 rpm. Partition efficiency for the DNP amino acids is substantially greater than that for the oligopeptides probably due to the lower viscosity of the chloroform phase system.

d) Separation of DNP amino acids and oligopeptides with a long column: The present method was applied for separation of DNP amino acids and oligopeptides with a long coiled column, The separation column was prepared from a 50 m long, 0.55 mm i.d. PTFE tube by winding it onto a 13 m long, 1.5 mm o.d.

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nylon pipe to make approximately 8500 helical turns with a total capacity of about 18 ml. The column was mounted on the column holder with a beta value of 0.75. Suitable sets of samples were selected each from DNP amino acids and oligopeptides for separation with the two-phase solvent systems used in the previous experiments.

In each separation, the column was first filled with the stationary phase and 5 ul of the sample solution containing each component at 0.05 to 0.6 g% was injected through the sample port. Then the mobile phase was pumped into the column at a flow rate of 2.4 ml/hr while the apparatus was run at revolutional speeds ranging between 400 and 600 rpm. The eluate was continuously monitored with an LKB Uvicord III at 280 nm.

In both DNP amino acid and oligopeptide separations, all components were well resolved under the elution with either aqueous or nonaqueous phase. DNP amino acid separation yielded higher partition efficiencies ranging between 6000 and 2000 theoretical plates. The oligopeptide separations showed skewing of the peaks due to the non-linear distribution isotherm which can be reduced by applying a lower concentration of the samples. The partition efficiencies for the oligopeptide separations range between 3000 and 300 theoretical plates.

The present method has been successfully applied to separations of a variety of biological samples such as gramicidine, calcitonine and various plant hormones.

Significance to Biomedical Research and the Program of the Institute:

The toroidal coil planet centrifuge is a compact tabletop model conveniently used in research laboratories. It enables separations of a variety of biological samples at a high partition efficiency without complication arising from the use of the solid supports. The capability of the present method may be further extended to separation of macromolecules and cells with polymer phase systems without complication caused by the use of rotating seals.

Proposed Course:

1. Application to separation of various biological materials with conventional two-phase solvent systems.

2. Application of the method to partition of macromolecules and cells with polymer phase systems.

3. Application of the method to elutriation of cells with a single phase physiological solution.

Publications:

1. Ito, Y.: Toroidal Coil Planet Centrifuge without Rotating Seals Applied to Countercurrent Chromatography. Anal. Biochem. 102, 150-152 (1980).

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2. Ito, Y.: The Toroidal Coil Planet Centrifuge for Countercurrent Chromatography. J. Chromatography 192 (No.1) 75-87, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 01438-01 LTD

PERIOD COVERED
October 1, 1979 through September 30, 1980

TITLE OF PROJECT (80 characters or less)

Large-Scale Preparative Countercurrent Chromatography with a slowly Rotating Coiled Tube

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Yoichiro Ito	Medical Officer	NHLBI	LTD

COOPERATING UNITS (if any)

LAB/BRANCH
Laboratory of Technical Development

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

An attempt to scale-up the preparative capacity of countercurrent chromatography was successfully made by the use of a large-bore glass coiled tube slowly rotating in the gravitational field. The capability of the present scheme was demonstrated in separations of DNP amino acids on a two-phase solvent system composed of chloroform, acetic acid, and 0.1N hydrochloric acid at 2:2:1 volume ratio. The method enables efficient separations of 600 mg samples in 10 hours with a good recovery. The present scheme is amenable to be further scaled-up by the use of longer and/or larger-diameter columns.

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PHS-6040
(Rev. 10-76)

Project Description: Development of large-scale preparative countercurrent chromatography with a slowly rotating coiled column.

Methods Employed and Major Findings:

a) Principle: When a coiled tube filled with a liquid is rotated around its horizontally oriented axis in the gravitational field, particles suspended in the liquid move toward one end of the coil. This end is called the head and the other, the tail of the coil. When the coil contains two immiscible solvent phases, the rotation soon establishes a hydrodynamic equilibrium state of the two phases. In this state the two phases are distributed in the coil in such a way that each phase occupies nearly equal space in each turn of the coil and any excess of either phase remains at the tail end of the coil while the two phases are constantly mixed by the rotation of the coil. This hydrodynamic behavior of the two phases can be efficiently used for solute partitioning. When the mobile phase is introduced at the head end of the coil to disrupt the hydrodynamic equilibrium state, the two phases quickly react to re-establish the original distribution pattern. This results in a countercurrent flow of the two phases in the coil: The stationary phase moves back toward the head end of the coil while the introduced excess amount of the mobile phase travels toward the tail. Consequently, the solutes introduced locally at the head of the coil are subjected to an efficient partition process and separated according to their partition coefficients as in liquid chromatography but in the absence of solid supports.

b) Apparatus: The motor drives the rotary frame, consisting of a pair of aluminum plates and links, around the horizontal stationary pipe mounted on the central axis of the apparatus. The rotary frame holds a pair of rotary shafts symmetrically spaced at 15 cm from the central axis of the apparatus. Each rotary shaft is equipped with a planetary gear which is engaged to an identical stationary sun gear mounted around the central stationary pipe. This gear coupling produces a synchronous planetary motion of each rotary shaft, i.e., the rotation about its own axis and revolution around the central axis of the apparatus in the same angular velocity in the same directions. This synchronous planetary motion of the rotary shaft eliminates the need for the rotating seal as reported earlier. In order to provide mechanical stability, a short coupling pipe was (coaxially mounted on the free end of the rotary frame and the other end of the coupling pipe was) supported by the stationary wall member of the apparatus through a ball bearing.

The separation column consists of 10 units of glass coiled tube of 0.5 cm i.d. and 2.5 cm core diameter (Kontes, NJ) connected in series (head to tail connection) with PTFE heat shrinkable tubing. The whole column has about 500 helical turns with a total capacity of approximately 900 ml. The column was symmetrically arranged around one of the rotary shifts by the aid of column supports. Counterweight was applied on the other rotary shafts to balance the apparatus. The flow tubes from the column were first passed through the center hole of the rotary shaft, led into the opening of the central stationary pipe through a side hole of the short coupling pipe and tightly supported at the exit hole of the stationary pipe. These tubes were lubricated with silicone grease and protected by a piece of plastic tubing to prevent direct contact with metal parts.

The apparatus can be operated at uniform rates ranging from 0 to 100 rpm, the limiting factor being the fragility of the glass coil. A Chromatronix Cherminert Pump or Beckman Accu Pump was used to pump the solvents and an LKB Uvicord III to monitor the absorbance of eluate at 280 nm.

c) Preliminary Studies on Partition Capability with a Short Column: Performance of the present scheme was evaluated by measuring the degree of stationary phase retention and partition efficiency in a short column consisting of 50 helical turns with a 90 ml total capacity. A two-phase solvent system composed of chloroform, acetic acid and 0.1 N hydrochloric acid at a volume ratio of 2:2:1 was used to partition a pair of DNP amino acids - 2,4,N-DNP-DL-glutamic acid and 2,4,N-DNP-L-alanine.

The percentage of stationary phase volume retained in the rotating coiled column relative to the total column capacity was determined at various revolutional speeds and flow rates. The results obtained with the stationary nonaqueous phase showed a satisfactory level of retention up to 20 rpm. The results with the stationary aqueous phase produced ideal levels of retention in a wide range of revolutional speeds and flow rates. This excellent retention of the aqueous phase may be caused by the affinity of the aqueous phase to the glass tube wall.

Studies on partition efficiency were performed under a wide range of revolutional speeds from 0 to 40 rpm at the flow rates of 120 ml/hr and 240 ml/hr where each phase was tested as the stationary phase. The partition efficiency sharply increased with the increase in revolutional speed from 0 to 20-30 rpm, where the peak resolution became maximum for both stationary phase groups. Further increase of the revolutional speed resulted in loss of peak resolution. The overall results indicated that the separation can be attained with either phase as the stationary phase.

d) Preparative Countercurrent Chromatography with a Long Column: The preparative capability of the present method was demonstrated on the separations of the same DNP amino acids with a long column consisting of 10 units, each identical to that used in the preliminary studies. The sample solution was prepared by dissolving 600 mg of an equal mixture of two components in 30 ml solvent. The separation was performed under the optimum operational condition of a 120 ml/hr flow rate and 30 rpm of revolutional speed by eluting with either nonaqueous or aqueous phase. The two DNP amino acids were completely separated within 10 hours in a high recovery of near 100%. The preparative capacity of the present method may be further increased by the use of larger-diameter and/or longer columns.

Significance to Biomedical Research and the Program of the Institute:

Separation and purification of a large amount of biological materials often becomes essential in biochemical and biomedical researches. The present method yields a highly efficient separation without complications arising from the use of solid supports. Thus, sample loss, denaturation and contamination are minimized.

Proposed Course:

1. Application of the method to separation of various biological samples.

2. Scaling-up of sample loading capacity.

Publication:

1. Ito, Y.: Preparative Countercurrent Chromatography with a Slowly Rotating Glass Coil. J. Chromatography in press

Annual Report of the
Pulmonary Branch
National Heart, Lung, and Blood Institute
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I. Basic Studies of the Control of Synthesis and Degradation of the Extracellular Matrix. Current concepts of the extracellular matrix of the alveolar structures suggest it is composed of four general classes of materials: (1) collagen, of which there are four types (types I and III are the interstitial collagens, types IV and V are the principle basement membrane collagens); (2) elastic fibers; (3) proteoglycans; and (4) glycoproteins (e.g., fibronectin) involved in cell-cell and cell-matrix interactions. In the past year, our studies have concentrated on the control of connective tissue production and destruction. The major findings in this area have been as follows.

(1) Regulation of collagen production by human fibroblasts. Human lung fibroblasts have been studied in detail concerning their regulation of collagen production. Normally these cells maintain very tight control over the amounts of collagen they produce. For example, collagen production during periods of rapid growth occurs at an identical rate as that during slow growth. However, even though these cells tightly regulate the amounts of collagen they can produce, they can modulate their collagen production. One important mechanism by which they do this is through intracellular degradation. Fibroblasts have mechanisms to "shunt" collagen from the normal secretory pathway to the lysosomal system. Detailed evaluation of this process has suggested that at least two "signals" can induce fibroblasts to increase intracellular collagen degradation. First, if the cell produces non-helical collagen, the intracellular degradation mechanisms recognize this and destroy this structurally abnormal collagen before it is secreted. Second, any signal that increases intracellular cyclic AMP will result in an increase in intracellular collagen degradation. Over the past year, methods have also been developed to quantitate messenger RNA levels and activities for type I collagen synthesis in cultured fibroblasts. Studies of cells during periods of rapid and slow growth have shown that there are significant alterations in mRNA levels although the activity of the mRNA (per molecule) is similar.

(2) Evaluation of cyclic AMP levels in human lung fibroblasts has demonstrated that this cyclic nucleotide is critically linked to collagen production. Agents that increase intracellular cyclic AMP (e.g., β-agonists, PEG$_1$ cholera toxin) all decrease collagen production by these fibroblasts. The concept has been developed that under normal conditions fibroblasts of the body are generally "suppressed" in terms of their potential for collagen production by endogenous β-stimulants. This suggests that the β-adrenergic system "holds back" collagen production and thus has the potential for significantly modifying the quantity of extracellular matrix components.

(3) Evaluation of collagen gene structure. In the past year, a major advance has been made in the evaluation of collagen gene structure through the isolation of a collagen gene using the methodologies of recombinant DNA. This collagen gene represents approximately 50% of the sheep pro α2 gene. It is one of the most complex and largest genes isolated to date. The structural information for 50% of the pro α2 mRNA is contained in 2.5 kilobases of DNA whereas 50% of the gene itself is approximately 17 kilobases. The vast majority of this gene is comprised of "introns" with only a small portion representing "exons" (the structural information). Evaluation of the structure of the sheep pro α2 gene using restriction enzymes and electron microscopic R loop mapping have shown that the exons are interspersed among the very large introns.

(4) Studies of collagen production is tendon, skin, and lung of sheep of varying gestational age have demonstrated that messenger RNA levels significantly control the production of collagen by these tissues. The percentage of protein synthesis devoted to collagen production varies in these tissues as a function of gestational age during fetal development, and appears to be paralleled, in general, by collagen messenger RNA levels. The exception is in skin, where high messenger RNA levels are maintained late in gestational life but the tissue does not use all the messenger RNA that is present.

(5) Studies of elastin production. Methods have been developed to quantitate the amount of elastin in various tissues as well as the rate of elastin synthesis by small pieces of tissue maintained in short term incubations. The technology has been developed to isolate and quantitate the elastin messenger RNA. Studies of these developing systems have shown that, in general, the rate of elastin production seems to be controlled by the amounts of available elastin mRNA in the tissue. In addition, elastin mRNA has been purified and a complementary DNA made to it. This cDNA is being used to probe tissues and cells for amounts of messenger RNA and to screen genomic "libraries" in order to isolate the elastin gene.

(6) Destruction of connective tissue components. Collagenase is an enzyme which specifically cleaves the collagen molecule at a point three-quarters of the distance from the N-terminal end. It has been thought that collagenase is the only neutral protease that has access to connective tissue that will attack collagen in the "collagenase" sensitive site. However, evaluation of human neutrophil elastase has shown that it can also act as a "collagenase" even though it has a broad spectrum of other proteolytic activities. Human neutrophil elastase will cleave type III collagen at a site close to the "collagenase" site. However, elastase does not attack type I collagen. In contrast, human neutrophil collagenase attacks type I collagen but not type III collagen.

II. Relationship of the Inflammatory and Immune Systems to the Maintenance of Lung Structure and Function. The inflammatory and immune systems of lung are important determinants of lung structure and function in health and disease. The Pulmonary Branch has a broad based program to evaluate the composition and function of the cellular and noncellular compoents of this system in animal models and in human lung. One major advance has been the development of methods to isolate inflammatory and immune effector cells and noncellular components from human lung by the technique of bronchoalveolar lavage, in which the fiberoptic bronchoscope is used to "wash out" a small segment of the epithelial surface of the lower respiratory tract. In the past year, these studies have included:

(1) Recruitment of inflammatory and immune effector cells to the lung. A number of acute and chronic lung disorders are associated with accumulation of neutrophils within the alveolar structures. Recent studies have demonstrated that the alveolar macrophage is capable of being stimulated to produce a chemotactic factor that is specific for neutrophils. Some of the stimuli that will induce alveolar macrophages to release this chemotactic factor include immune complexes, particulates, and infectious organisms. The chemotactic factor is of low molecular weight and predominently lipid in nature. Other studies have demonstrated that fragments of elastin are capable of attracting monocytes to the lung parenchyma. Since a variety of human disorders are associated with destruction of elastin fibers, this gives a mechanism for the accumulation of mononuclear phagocytes within the lung parenchyma.

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(2) Animal models. An animal model of hypersensitivity pneumonitis has been established using animals sensitized to ovalbumin and then given intratracheal particulate ovalbumin. The initial phase of the pulmonary inflammatory response is the accumulation of neutrophils within the lung parenchyma mediated by release of neutrophil chemotactic factor by alveolar macrophages. Subsequently, there are shifts in the lymphocyte cell populations such that T-lymphocytes are increased in proportion, likely an important mechanism for the subsequent formation of granuloma. An animal model of bleomycin administration has been used to induce pulmonary fibrosis within mice. By using a variety of mice with genetic abnormalities, it has been possible to demonstrate that T-lymphocytes are not necessary for the development of the fibrotic lesion. An animal model of asbestosis has been established; the initial reaction is an accumulation of neutrophils within the alveolar structures. The mechanism is mediated by production of neutrophil chemotactic factor by macrophages in which the asbestos particle induces the macrophage to release the chemotactic factor. A new hereditary animal model of emphysema has been discovered in the "tight skin" mouse. These animals have skin that is similar to the human disease scleroderma. In their lungs, however, they have morphologic and physiologic abnormalities identical to that of human panacinar emphysema. An animal model of hereditary interstitial lung disease has been discovered in the "moth-eaten" mouse; this represents the first hereditary disorder in animals of interestitial lung disease.

(3) Oxidant injuiry to the lung. Methods have been developed to directly evaluate oxidant injury to the lung parenchyma of animals and humans. Pieces of lung are labeled with ^{51}Cr, a classic method for evaluating cell cytotoxicity. Small pieces of these labeled explants are then incubated with various mediators or cells thought to initiate oxidant injury. Some of the injurious methods used include high concentrations of oxygen, paraquat (a herbacide the injection of which is associated with fatal lung damage), and a variety of inflammatory and immune effector cells known to produce oxidants.

(4) Growth factors. One mechanism to explain the fibrosis is the interstitial lung diseases is that the collagen producing cells of the lung are increased in number even though they may be producing the same number of collagen molecules per cell. To evaluate the hypothesis that the alveolar macrophage may produce a growth factor that induces human fibroblasts to replicate, a variety of stimuli have been used to activate alveolar macrophages in guinea pigs or humans. Supernatants from these macrophages have been added to human lung fibroblasts in culture and the number of cells counted as a function of time. A growth factor has been identified for both animal and human alveolar macrophages. In addition, particulates such as asbestos will induce the alveolar macrophage to produce this growth factor thus explaining one of the mechanisms of fibrosis in this disease. In preliminary experiments, human alveolar macrophages have been evaluated from patients with a variety of interstitial lung diseases; these studies suggest that in conditions in which the macrophage is activated they are producing this growth factor. This may represent an important mechanism of fibrosis in human lung disorders and a possible site of intervention for the therapy of these diseases.

III. Clinical Studies of Lung Disease. The clinical studies of the Pulmonary Branch involve three general categories of chronic lung disorders: interstitial lung disease destructive lung disease, and hereditary lung disease.

The interstitial lung disorders represent 15-30% of the noninfections disorders of lung. In the past year, the Pulmonary Branch has continued its detailed studies of patients with interstitial disease with particular emphasis on those with idiopathic pulmonary fibrosis (IPF) and sarcoidosis. The destructive lung disorders are disorders in which the alveolar structures are lost. Current concepts of the pathogenesis of these disorders is defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in the normal lung there is a balance between proteases (e.g., elastase) released by inflammatory and immune effector cells within the lung balanced by anti--proteases (e.g., α1-antitrypsin, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases, presumably there is an imbalance such that the proteases overpower the anti-protease systems, thus causing connective tissue destruction and loss of portions of the alveolar structures. During the past year, the interest of the Pulmonary Branch in the destructive lung disorders has been directed toward evaluating the influence of smoking on destruction in the lower respiratory tract and evaluation of the pathogenesis and therapy of α1-antitrypsin deficiency (a hereditary disorder associated with panacinar emphysema). There are several forms of lung disease which have a known hereditary basis. By evaluating these "experiments of nature" we can derive an understanding of the structure and function of the lung which can then be applied to more general understanding of pulmonary disorders. In addition to α1-antitrypsin deficiency, several of these disorders have been chosen as models of more generalized forms of lung disease.

(1) Pathogenesis and therapy of interstitial pulmonary fibrosis. Bronchoalveolar studies of patients with IPF have demonstrated that approximately 70% of these individuals have active collagenase in their lower respiratory tract. Since the lung possesses no anti-collagenase, this active enzyme likely plays an important role in deranging the connective tissue matrix. Detailed evaluation of the colagenase obtained from the lungs of these patients has shown that it is derived from the neutrophil. Lavage studies have also demonstrated that these neutrophils are attracted to the lungs of these patients in a chronic fashion by the presence of immune complexes in the lower respiratory tract. The immune complexes interact with alveolar macrophages to induce it to produce a chemotactic factor specific for neutrophils. Although the antigen in this disease is not clear, approximately 25% of the patients are producing IgG in the lung directed against type I collagen. Evaluation of immunoglobulin production by β-lymphocytes in the lungs of these patients show that they are producing approximately 150 times more immunoglobulin per 10^6 lymphocytes than are normal individuals. In addition, this disease is localized to lung; evaluation of β-lymphocytes in blood and bone marrow of these patients has shown that the elevated immunoglobulin production is specific for lung β-lymphocytes. Prospective evaluation of the level of neutrophils in the lungs of these patients has shown that those patients with high proportions of neutrophils deteriorate even on therapy whereas individuals with low proportions of neutrophils stabilize or improve. A randomized protocol was carried out in which individuals were either treated with oral prednisone alone or with oral prednisone together with high dose intravenous corticosteroids given on a once a week basis (2 grams solu-medrol intravenously). Evaluation of the neutrophil burden in the lungs of the two groups after four months demonstrated that those on high dose corticosteroids had less inflammation in the lung than those treated with oral prednisone alone.

(2) Pathogenesis and therapy of sarcoidosis. Evaluation of the inflammatory and immune effector cells in the lungs of patients with sarcoidosis has demonstrated they comprise high concentrations of activated T-lymphocytes. One of the products produced by these activated T-lymphocytes is monocyte chemotactic factor; thus providing a mechanism for accumulation of monocytes to the lungs of these patients. Since monocytes are the building blocks for granuloma formation this likely provides the mechanism by which granuloma are maintained in the sarcoid lung. It has been known for some time that patients with sarcoidosis have hypergammaglobulinemia even though B-lymphocytes in the peripheral blood are producing very little immunoglobulin. Evaluation of the B-lymphocytes of the lungs of patients with sarcoidosis has shown that the high immunoglobulins in the blood is likely derived from the lung. In addition, it appears that the elevated immunoglobulin production by lung lymphocytes in sarcoidosis patients is secondary to a polyclonal stimulation of lung B-lymphocytes by activated lung T-lymphocytes. Prospective evaluations of patients with sarcoidosis have revealed that quantitation of T-lymphocytes in the lung together with quantitation of ^{67}gallium scans as an effective means to stage inflammation in these patients. If a sarcoid patient has high intensity alveolitis (greater than 28% T-lymphocytes plus a positive gallium scan), 100% of these will deteriorate in at least one functional parameter over the subsequent six months. In contrast, if the individual has low intensity alveolitis (T-lymphocytes less than 28% and/or negative gallium scan) there is more than 80% chance the patient will stabilize or will improve over the following six months.

(3) Pathogenesis and therapy of destructive lung disease. Alpha 1-antitrypsin is the major antielastase of the lower respiratory tract. In cigarette smokers α1-antitrypsin levels in lung are normal. However, functional evaluation of the α1-antitrypsin in the alveolar structures has shown that it is at least 50% functionally deficient (i.e., it is present but not working properly). The likely mechanism for this is that cigarette smoke, either by itself or through inflammatory and immune effector cells, oxidize a methionine group near the active site of this antiprotease. Direct evaluation of lung biopsies of smokers has shown that they have neutrophils within the lung. Since these cells carry elastase and other proteolytic enzymes, this is likely a critical mechanism for the destruction of lung in this disease. A detailed evaluation has been carried out of the protease-antiprotease balance within the lower respiratory tract of normal individuals and individuals with α1-antitrypsin deficiency. Whereas α1-antitrypsin is the major antielastase of normal individuals, individuals with a deficiency of this enzyme in the serum have no antielastase protection in the lower respiratory tract. Two therapeutic protocols have been carried out for destructive lung disease, both involving patients with homozygous PiZ α1-antitrypsin deficiency. The impeded androgen Danasol has been used for one month period in a group of patients; in the vast majority, a1-antitrypsin levels increased approximately 50%. While this does not cure the disease, it does bring the α1-antitrypsin levels closer to the theoretical protective threshold (80 mg/dl) of serum α1-antitrypsin. A direct therapeutic approach to α1-antitrypsin deficiency has been taken in which 5 patients were treated with intravenous replacement of α1-antitrypsin derived from pooled plasma of normal individuals. Each individual was given 4 gm of α1-antitrypsin intravenously over an 8 hour period once a week for 4 weeks. In all 5, the levels achieved for the one month period were consistently above 80 mg/dl. In addition, direct evaluation of the antiprotease screen of the lower respiratory tract of these patients has shown that whereas they were devoid of antielastase protection before therapy, after intravenous α1-antitrypsin infusions, they had adequate lung antielastase protection.

(4) Scintigraphic evaluation of patients with chronic lung disease. Gallium scans have continued to play an important role in evaluation of chronic inflammatory disease. This technique is gaining wide acceptance since its use was pioneered by the Department of Nuclear Medicine, Clinical Center in conjunction with the Pulmonary Branch. Gallium scans have been evaluated both in idiopathic pulmonary fibrosis and sarcoidosis in a prognosis use in determining which patients will deteriorate. Together with bronchoalveolar lavage this is a useful, nonevasive method to evaluate these patients. A new method has been developed by the Nuclear Medicine Department by which dynamic ventilation scans can be carried out both at rest and in exercise. These scans utilize ^{127}Xe in inhalation and then study the lung as a function of time. "Movies" can be made of the lung "breathing." Preliminary use of this method has suggested that it may be very useful in evaluating a variety of lung disorders such as destructive lung disease.

(5) Morphologic evaluation of fibrotic lung. Electron microscopic evaluation of upper lung biopsies from patients with interstitial lung disease have been evaluated in collaboration with the Pathology Branch, NHLBI and Dr. Francoise Basset, Hospital Bichat, University of Paris (through a cooperative agreement with INSERM and NIH). These studies have demonstrated a number of new findings including: (a) nuclear inclusions in epithelial cell nuclei; (b) accumulation of mast cells in the alveolar structures of patients with fibrotic lung disease; (c) a new structure, "anchoring fibrils", which are found in normal bronchi but not in alveolar structures and are present in large abundance beneath the basement membrane of patients with fibrotic lung disease; (d) evaluation of the derivation of the epithelial cells in fibrotic lung disease to demonstrate that they are not only derived from the existing epithelial cells but also from the bronchiolar epithelium; and (e) the evaluation of so-called "Langerhan's cells" in the lungs of patients with fibrotic lung disease, particularly those with histiocytosis-X.

(6) Physiologic studies. Detailed evaluation of Fabry's disease (Angiokeratoma Corporis Diffusion Universlae) a sex-linked sphingolipid disorder, has shown that these patients have airway disease. The functional abnormalities to limitation of the airflow is correlated with morphological findings of deposits of ceramide trihoxoside in epithelial cells of the bronchi of these patients. Functional evaluation of patients with Wegener's granulomatosis have demonstrated that they have functional abnormalities in volume, flow rates, and diffusing capacity. Whereas the first two seem to improve with therapy the diffusing capacity may continue to deteriorate. Functional parameters may be useful to follow these patients in addition to X-ray.

(7) Connective tissue changes in fibrotic lung disease. Evaluation of collagen concentration and rates of collagen synthesis in patients with idiopathic pulmonary fibrosis have demonstrated that both are normal. Although this may seem suprising and not consistent with the morphology, it suggests that the fibrotic process is much more complex than originally thought and likely involves alterations not only in the amount but also location, type and form of connective tissue. Evaluation of bronchoalveolar lavage of patients with fibrotic lung disease have shown that they have elevations of fibronectin, a connective tissue macromolecule known to mediate cell-cell and cell-matrix interactions. Normal individuals have the same levels of fibronectin in lavage fluid as in serum, suggesting that the elevation in the lungs of patients with fibrotic lung disease may be associated with the inflammatory process which destroys the fibronectin within the lung.

(8) Hereditary studies of connective tissue. Studies have continued to evaluate the lung function of patients with hereditary disorders of connective tissue such as osteogenesis imperfecta and Ehlers-Danlos syndrome (particularly type IV). Most of the data to data suggest that lung function in these individuals is normal. Since it is known that each of these patients studied have marked abnormalities in connective tissue in other parts of the body this would suggest that there may be more than one gene for each collagen type. For example, skin biopsies of patients with Ehlers-Dnalos syndrome have a deficiency of type III collagen, if type III collagen is important in lung function (which it is thought to be) then it is unlikely that the same gene is operating in the lung parenchyma.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02405-07 PB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)
Relationship of Inflammatory and Immune Systems to the Maintenance of Lung Structure and Function

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Crystal	Chief, Pulmonary Branch	NHLBI PB
Other:	G. Hunninghake	Staff Investigator	NHLBI PB
	J. Gadek	Staff Investigator	NHLBI PB
	K. Bradley	Chemist	NHLBI PB
	G. Fells	Biologist	NHLBI PB
	R. Zimmerman	Biologist	NHLBI PB
	B. Keogh	Staff Investigator	NHLBI PB
	S. Szapiel	Biologist	NHLBI PB
	C. Schoenberger	Clinical Associate	NHLBI PB
	P. Bitterman	Clinical Associate	NHLBI PB
	W. Martin	Guest Worker	NHLBI PB
	R. Haber	Biologist	NHLBI PB

COOPERATING UNITS (if any)
None

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 9.3	PROFESSIONAL: 6.3	OTHER: 3.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

It is clear that the inflammatory and immune systems are critically important for the maintenance of lung structure and function in health and disease. The Pulmonary Branch has instituted a broad-based program to evaluate the composition and function of the cells comprising these systems in animal models and in both normal and diseased human lung. Methods have been developed to define these cell populations and evaluate their effector function. Particular attention is being paid to macrophage production of factors chemotactic for neutrophils, influence of immune effector cells on connective tissue production by mesenchymal cells, evaluation of the inflammatory and immune effector cells comprising the alveolitis of idiopathic pulmonary fibrosis and sarcoidosis, and animal models of hypersensitivity pneumonitis.

PHS-6040
(Rev. 10-76)

Other:	P. Broska	Bio Lab Tech	NHLBI PB
	B. Davis	Staff Investigator	NHLBI PB
	E. Moritz	Staff Investigator	NHLBI PB
	G. Rossi	Visiting Associate	NHLBI PB

Project Description:

Objectives: The vast number of chronic disorders of the alveolar structures are associated with an alveolitis, i.e., the accumulation of inflammatory and immune effector cells within the alveolar interstitium and alveolar air spaces. In the past year the Pulmonary Branch has continued a broad based program to evaluate the composition and effector function of the alveolitis of these disorders. Our objectives in the past year have been to: (1) develop animal models of interstitial lung disease in which the influence of the inflammatory and immune systems can be evaluated; (2) evaluation of the mechanisms of inflammatory and immune effector cell traffic into the alveolar structures; (3) evaluation of the influence of immune effector cells on extracellular matrix macromolecule production by mesenchymal cells; and (4) evaluation of the immune and inflammatory effector cells comprising the alveolitis of the human interstitial lung disorders (see also Project No. Z01 HL 02407-06 PB).

Methods: The techniques used for these studies are standard in the fields of inflammation and immunology. For animal studies of interstitial lung disease two stimuli were used: (1) bleomycin, an anti-tumor antibiotic known to cause interstitial lung disease in 10% of patients receiving it; (2) particulate ovalbumin in previously sensitized animals; the particulate ovalbumin is used as an analogue of antigens in the hypersensitivity lung disorders. For the bleomycin studies, mice are utilized that have carefully defined genetic strains lacking a component of the immune system. For the hypersensitivity studies, inbred strains of guinea pigs are utilized. To evaluate the mechanism of the inflammatory and immune effector cell traffic into the alveolar structures, alveolar macrophages derived from the guinea pig lung were cultured *in vitro* with various agents used as analogues of stimuli causing human disorders. In addition, alveolar macrophages from normal human volunteers were utilized *in vitro* in similar studies. In both of these situations, various target cells were used to evaluate the possible chemotactic factors produced by the alveolar macrophages under various states of stimulation. To evaluate the inflammatory and immune effector cells comprising the alveolitis of the human interstitial lung disorders (see also Project Z01 HL 02407-06 PB), two methodologies were utilized: (1) bronchoalveolar lavage via the fiberoptic bronchoscope; and (2) open lung biopsy. In both cases, inflammatory and immune effector cells were recovered, purified by gradient centrifugation and subtypes of the cells purified by conventional means. These cell populations were then evaluated for types of cells present and effector function of the cells.

Major Findings: In the past year, these studies have included:

(1) Recruitment of inflammatory and immune effector cells to the lung. A number of acute and chronic lung disorders are associated with accumulation of neutrophils within the alveolar structures. Recent studies have demonstrated that the alveolar macrophage is capable of being stimulated to produce a chemotactic factor that is specific for neutrophils. Some of the stimuli that will induce alveolar macrophages to release this chemotactic factor include immune complexes, particulates, and infectious organisms. This phenomenon seems to be similar across species lines including humans. The chemotactic factor is of low molecular weight and predominently lipid in nature. However, it is distinct from all other chemotactic factors including the lipid chemotactic factors produced by mast cells. Other studies have demonstrated that fragments of elastin are capable of attracting monocytes to the lung parenchyma. Since a variety of human disorders are associated with destruction of elastin fibers, this gives a mechanism for the accumulation of mononuclear phagocytes within the lung parenchyma. Evaluation of the elastin fragments suggested that elastin cross-links are necessary compliments to provide the chemotactic stimulus.

(2) Animal models. An animal model of hypersensitivity pneumonitis has been established using animals sensitized to ovalbumin and then given intratracheal particulate ovalbumin. The initial phase of the pulmonary inflammatory response is the accumulation of neutrophils within the lung parenchyma. This is mediated by production of neutrophil chemotactic factor by alveolar macrophages.

Subsequently, there are shifts in the lymphocyte cell populations such that T-lymphocytes are increased in proportion. This is likely an important mechanism for the subsequent formation of granuloma in these disorders. An animal model of bleomycin administration has been used to induce pulmonary fibrosis within mice. By using a variety of mice with genetic abnormalities, it has been possible to demonstrate that T-lymphocytes are not necessary for the development of the fibrotic lesion. An animal model of asbestosis has been established. In humans it has been demonstrated that there are neutrophils in the lungs of patients with asbestosis. In this animal model in guinea pigs the initial reaction is also an accumulation of neutrophils within the alveolar structures. The mechanism also seems to be mediated by production of neutrophil chemotactic factor by the alveolar macrophage in which the asbestos particle induces the macrophage to release the chemotactic factor. A new hereditary animal model of emphysema has been discovered in the so-called "tight skin" mouse. These animals have skin that is similar to the human disease scleroderma. In their lungs, however, they have morphologic and physiologic abnormalities identical to that of human panacinar emphysema. Preliminary evaluation of the pathogenesis of the lung lesion suggests that there is an accumulation of inflammatory and immune effector cells including polymorphonuclear leukocytes. Evaluation of the serum antiproteases in these animals suggest they have an adequate antielastase. Thus, if a protease-antiprotease imbalance is responsible for the pathogenesis of emphysema in the tight skin mouse it would have to be due to a protease excess rather than antiprotease deficiency. An animal model of hereditary interstitial lung disease has been discovered in the "moth-eaten" mouse. This represents the first hereditary disorder in animals of interestitial lung disease. The model is a complex one as there are a variety of complex inflammatory and immune processes ongoing both within the lung and systemically in these animals. However, they die of pulmonary insufficiency with alveolitis and fibrosis.

(3) Oxidant injury to the lung. Methods have been developed to directly evaluate oxidant injury to the lung parenchyma of animals and humans. Pieces of lung are taken and labeled with ^{51}Cr, a classic method for evaluating cell cytotoxicity. Small pieces of these labeled explants are then incubated with various mediators or cells thought to initiate oxidant injury. Some of the injurious methods used include high concentrations of oxygen, paraquat (a herbacide the injection of which is associated with fatal lung damage), and a variety of inflammatory and immune effector cells known to produce oxidants. This model system has easy adaptability to evaluation of drugs that may protect the lung from oxidant injury.

(4) Growth factors. One mechanism to explain the fibrosis in the interstitial lung diseases is that the collagen producing cells of the lung are increased in number even though they may be producing the same number of collagen molecules per cell. To evaluate the hypothesis that the alveolar macrophage may produce a growth factor that induces human fibroblasts to replicate, a variety of stimuli have been used to activate alveolar macrophages in guinea pigs or humans. Supernatants from these macrophages have been added to human lung fibroblasts in culture and the number of cells counted as a function of time. A growth factor has been identified for both animal and human alveolar macrophages. In addition, particulates such as asbestos will induce the alveolar macrophage to produce this growth factor thus explaining one of the mechanisms of fibrosis in this disease. In preliminary experiments, human alveolar macrophages have been evaluated from patients with a variety of interstitial lung diseases; these studies suggest that in conditions in which the macrophage is activated they are producing this growth factor. This may represent an important mechanism of fibrosis in human lung disorders and a possible site of intervention for the therapy of these diseases.

(5) Anti-thymocyte cross-reactivity. Anti-thymocyte globulin is a frequently used reagent in helping individuals to accept transplants of organs. One of the problems in these patients is a pneumonitis, generally thought to be due to

infection. However, evaluation of commercially available anti-thymocyte globulin has shown that it cross-reacts with a variety of human lung cells including human lung fibroblasts and human alveolar macrophages. Thus, one mechanism by which inflammatory lung disease occurs in these individuals is by a side effect of the therapeutic agent rather than secondary infection.

Significance to Biomedical Research and the Program of the Institute: The hypothesis that the inflammatory and immune systems are important factors in modulating the development of interstitial lung disease is becoming a proven reality. It is likely that if it is possible to effectively treat these disorders, the most vulnerable complement in the pathogenesis will be at this step. A definition of the cells comprising the alveolitis of these disorders as well as the function of the cells comprising it, are critical steps in understanding the process involved in the production of interstitial lung disease.

Proposed Course: Studies will continue to utilize animal models as well as cells obtained from humans, to evaluate the role of the inflammatory and immune systems in the maintenance of alveolar structure in health and disease.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF** **INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02407-06 PB

PERIOD COVERED

October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Clinical Studies of Lung Disease

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Crystal	Chief, Pulmonary Branch	NHLBI PB
Other:	G. Hunninghake	Staff Investigator	NHLBI PB
	J. Gadek	Staff Investigator	NHLBI PB
	I. Strumpf	Staff Investigator	NHLBI PB
	B. Keogh	Staff Investigator	NHLBI PB
	C. Schoenberger	Clinical Associate	NHLBI PB
	P. Bitterman	Clinical Associate	NHLBI PB
	S. Rennard	Staff Investigator	NHLBI PB
	B. Davis	Staff Investigator	NHLBI PB
	E. Moritz	Staff Investigator	NHLBI PB
	G. Rossi	Visiting Associate	NHLBI PB
	K. Bradley	Chemist	NHLBI PB

COOPERATING UNITS (if any)

B. Line	Staff Investigator	CR LAS
A. Nienhuis	Chief, Clinical Hematology Branch	NHBLI CL

LAB/BRANCH

Pulmonary Branch

SECTION

INSTITUTE AND LOCATION

NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 12.4	PROFESSIONAL: 8.0	OTHER: 4.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The interstitial lung disorders represent 15 to 20% of all pulmonary disorders; in most cases these diseases cause significant disability and many are fatal. Studies of the natural history, etiology, pathogenesis, pathophysiology and therapy of these disorders have made major inroads into understanding these diseases. Most importantly is the development of the concept that the inflammatory and immune effector cells are critical determinants in the pathogenic process. Methodologies have been developed to evaluate the alveolitis of these patients and to examine its effect on the alveolar structures, particularly the extracellular matrix. Therapeutic trials are underway to evaluate the efficacy of drug programs aimed at irradicating the alveolitis of these diseases. Smoking induces macrophages to produce chemotactic factors for neutrophils. In addition, cigarette smoke likely reduces the ability of α1-antiproteinase to function normally. Therapeutic trials are ongoing for treatment of α1-antitrypsin deficiency using danazol therapy to increase release of the anti-protease from the liver and a direct replacement trial of α1-antiproteinase is ongoing.

PHS-6040
(Rev. 10-76)

Other:	G. Fells	Biologist	NHLBI PB
	R. Zimmerman	Biologist	NHLBI PB
	P. Broska	Bio Lab Tech	NHLBI PB

Cooperating Units:

O. Kawanami	Visiting Scientist	NHLBI PA
V. Ferrans	Chief, Ultrastructure Section	NHLBI PA
W. Roberts	Chief, Pathology Branch	NHLBI PA
A. Fauci	Chief, Clinical Physiology Section	I LCI
R. Brady	Chief, Developmental and Metabolic Neutrology Branch	N DMN
M. Frank	Chief, Laboratory of Clinical Investigation	I LCI

Project Description:

Objectives: The interstitial lung disorders represent 15 to 30% of the non-infectious disorders of the lung. There are more than 130 separable disease entities associated with interstitial disease, but they can be categorized into two general groups: those of known etiology and those of unknown etiology. The interstitial lung diseases of known etiology include the occupational and environmental inhalents (inorganic dust, organic dust, gases, fumes, vapors, aerosols), drugs, poisons, radiation, and interstitial disease caused by disorders of organs other than lung. The interstitial lung diseases of unknown etiology include more than 20 different categories, the most important of which are idiopathic pulmonary fibrosis (IPF), chronic interstitial disease associated with the collagen vascular disorders, sarcoidosis, and eosinophilic granuloma. The Pulmonary Branch has undertaken a detailed study of patients with interstitial disease, particularly those with IPF, sarcoidosis and interstitial disease caused by inorganic and organic dusts. IPF has been of particular interest as it represents a chronic devastating illness resulting in death an average of 4 to 5 years from the onset of symptoms. Although approximately 5 to 10% of patients with IPF respond to corticosteroids, there is no known treatment for the remainder. Although sarcoidosis is a less devastating illness for most patients with the disease, it represents a more common form of interstitial lung disease, occurring in 40/100,000 blacks in this country. Of those, 5 to 11% will eventually die of the disease and up to 30% are disabled by pulmonary insufficiency. The objectives of our studies are to define the natural history, etiology, pathogenesis, pathophysiology, and therapy of the interstitial lung diseases. In the past year, we have concentrated primarily on IPF and sarcoidosis.

The destructive lung disorders are disorders in which the alveolar structures are lost. Current concepts of the pathogenesis of these disorders is defined by the so-called "protease-antiprotease" theory of emphysema. This theory holds that in the normal lung there is a balance between proteases (i.e., elastase) released by inflammatory and immune effector cells both within, and trafficking to the lung, balanced by anti-proteases (i.e., α1-antiproteinase, a circulating antiprotease that diffuses into the lung). In the destructive lung diseases, presumably there is an imbalance such that the proteases overpower the anti-protease systems, thus causing connective tissue destruction and loss of portions of the alveolar structures. During the past year, the interest of the Pulmonary Branch in the destructive lung disorders has been directed toward the influence of smoking on destruction in the lower respiratory tract, evaluation of the therapy of α1-antitrypsin deficiency (a hereditary disorder associated with panacinar emphysema), and various other clinical studies of lung diseases associated with destruction of the alveolar structures.

There are several forms of lung disease which have a known hereditary basis. By evaluating these "experiments in nature" we can derive an understanding of the structure and function of the lung which can then be applied to more general understanding of pulmonary disorders. In addition to α1-antitrypsin deficiency discussed above, Fabry's Disease and hereditary disorders of connective tissue have also been studied.

Methods: Patients admitted to the Pulmonary Branch Clinical Service enter an extensive protocol which includes: detailed medical and pulmonary history and physical exam, routine serologic, roentgenographic and EKG studies, serologic studies aimed at immune processes; pulmonary function studies including lung volumes, flow rates, diffusing capacity, flow-volume curves, closing volume, closing capacity, body plethysmography for functional residual volume and airway resistance, static and dynamic pressure-volume curves, ventilatory and arterial blood gas studies at rest and exercise, lung lavage for cellular function and morphology as well as non-cellular constituents of the lower respiratory tract, ventilation and perfusion scans, and gallium 67 scans. In some patients, the pulmonary circulation is evaluated with right heart catheterization at rest and exercise. When indicated lung biopsy is done either thru the fiberoptic bronchoscope or via open thoracotomy. Tissues are studied by light microscopy, electron microscopy culture, evaluation of the cellular components of the inflammatory and immune systems and evaluation of the constitutents of the extracellular matrix. Selected patients are entered into drug treatment protocols aimed at halting the alveolitis of the disease.

Major Findings:

(1) Pathogenesis and therapy of interstitial pulmonary fibrosis. Bronchoalveolar studies of patients with idiopathic pulmonary fibrosis (IPF) have demonstrated that approximately 70% of these individuals have active collagenase in their lower respiratory tract. Since the lung possesses no anti-collagenase, this active enzyme likely plays an important role in deranging the connective tissue matrix. Detailed evaluation of the collagenase obtained from the lungs of these patients has shown that it is derived from the neutrophil. Lavage studies have also demonstrated that these neutrophils are attracted to the lungs of these patients in a chronic fashion by the presence of immune complexes in the lower respiratory tract. The immune complexes interact with alveolar macrophages to induce it to produce a chemotactic factor specific for neutrophils. Although the antigen in this disease is not clear, approximately 25% of the patients are producing IgG in the lung directed against type I collagen. Evaluation of immunoglobulin production by B-lymphocytes in the lungs of these patients show that they are producing approximatley 150 times more immunoglobulin per 10^6 lymphocytes than are

normal individuals. In addition, this disease is localized to lung; evaluation of B-lymphocytes in blood and bone marrow of these patients has shown that the elevated immunoglobulin production is specific for lung B-lymphocytes. Prospective evaluation of the level of neutrophils in the lungs of these patients in terms of functional status one year later has shown that those patients with high proportions of neutrophils deteriorate even on therapy whereas individuals with low proportions of neutrophils stabilize or improve. A double-blind study has been completed in which all patients with IPF have been treated with corticosteroids and 50% (in a double-blind fashion) treated with azathioprine. Evaluation of the functional status of these patients after one year demonstrated no difference between the two groups. However, those on azathioprine had higher incidences of drug toxicity. A randomized protocol was also carried out in which individuals were either treated with oral prednisone alone or with oral prednisone together with high dose intravenous corticosteroids given on a once a week basis (2 grams solumedrol intravenously). Evaluation of the neutrophil burden in the lungs of the two groups after four months demonstrated that those on high dose corticosteroids clearly had less inflammation in the lung than those treated with oral prednisone alone.

(2) Pathogenesis and therapy of sarcoidosis. Evaluation of the inflammatory and immune effector cells in the lungs of patients with sarcoidosis has demonstrated they have high concentrations of T-lymphocytes and that these T-lymphocytes are activated. One of the products produced by these activated T-lymphocytes is monocyte chemotactic factor; thus providing a mechanism for accumulation of monocytes to the lungs of these patients. Since monocytes are the building blocks for granuloma formation this likely provides the mechanism by which granuloma are maintained in the sarcoid lung. It has been known for some time that patients with sarcoidosis have hypergammaglobulinemia even though B-lymphcoytes in the peripheral blood are producing very little immunoglobulin. Evaluation of the B-lymphocytes of the lungs of patients with sarcoidosis has shown that the high immunoglobulins in the blood is likely derived from the lung. In addition, it appears that the elevated immunoglobulin production by lung lymphocytes in sarcoidosis patients is secondary to a polyclonal stimulation of lung B-lymphocytes by activated lung T-lymphocytes. Several investigators have proposed that angiotensin converting enzyme is an useful method to evaluate the activity of sarcoidosis. However, correlation of serum angiotensin converting enzyme levels in the blood of these patients with the proportions of T-lymphocytes in the lung have demonstrated that there are both false positives and false negatives of serum angiotensin converting enzyme. Prospective evaluations of patients with sarcoidosis have revealed that quantitation of T-lymphocytes in the lung together with quantitation of ^{67}gallium scans as an effective means to stage inflammation in these patients. If a sarcoid patient has high intensity

alveolitis (greater than 28% T-lymphocytes plus a positive gallium scan), 100% of these will deteriorate in at least one functional parameter over the subsequent six months. In contrast, if the individual has low intensity alveolitis (T-lymphocytes less than 28% and/or negative gallium scan), there is more than 80% chance the patient will stabilize or will improve over the following six months.

(3) Pathogenesis and therapy of destructive lung disease. Alpha 1-antitrypsin is the major antielastase of the lower respiratory tract. In cigarette smokers α1-antitrypsin levels in lung are normal. However, functional evaluation of the α1-antitrypsin in the alveolar structures has shown that it is at least 50% functionally deficient (i.e., it is present but not working properly). The likely mechanism for this is that cigarette smoke, either by itself or through inflammatory and immune effector cells, oxidize a methionine group near the active site of this antiprotease. Direct evaluation of lung biopsies of smokers has shown that they have neutrophils within the lung. Since these cells carry elastase and other proteolytic enzymes, this is likely a critical mechanism for the destruction of lung in this disease. A detailed evaluation has been carried out of the protease-antiprotease balance within the lower respiratory tract of normal individuals and individuals with α1-antitrypsin deficiency. Whereas α1-antitrypsin is the major antielastase of normal individuals, individuals with a deficiency of this enzyme in the serum have no antielastase protection in the lower respiratory tract. Two therapeutic protocols have been carried out for destructive lung disease, both involving patients with homozygous PiZ α1-antitrypsin deficiency. The impeded androgen Danazol (2,3-isoxazol-17 α-ethinyl testosterone) has been used for over a one month period in a group of patients. In the vast majority, α1-antitrypsin levels increased approximately 50%. While this does not cure the disease, it does bring the α1-antitrypsin levels closer to the theoretical threshold (80 mg/dl) of serum α1-antitrypsin, enough to protect the lung from proteolytic damage. A direct therapeutic approach to α1-antitrypsin deficiency has been taken in which 5 patients were treated with intravenous replacement of α1-antitrypsin derived from pooled plasma of normal individuals. Each of the 5 individuals was given 4 gm of α1-antitrypsin intravenously over an 8 hour period once a week for 4 weeks. In all 5 individuals the levels achieved for the one month period were consistently above 80 mg/dl. In addition, direct evaluation of the antiprotease screen of the lower respiratory tract of these patients has shown that whereas they were devoid of antielastase protection before therapy, after intravenous α1-antitrypsin infusions, they had adequate antielastase protecion in the lower respiratory tract.

(4) Evaluation of inflammatory and immune effector cells in the lower respiratory tract. The technique of bronchoalveolar lavage has been widely used to

evaluate inflammatory and immune effector cells in the alveolar structures. To directly evaluate the concept that the bronchoalveolar lavage actually samples inflammatory and immune effector cells of the alveolar structures, bronchoalveolar lavage inflammatory and immune effector cells were compared to those derived directly from open lung biopsies in the same patients. The spectrum of inflammatory and immune effector cells was found to be almost identical in a variety of diseases including idiopathic pulmonary fibrosis and sarcoidosis as well as controls. Thus it appears that bronchoalveolar lavage is a valid means by which to assess the inflammatory and immune effector cells in the lower respiratory tract of patients with chronic interstitial disease.

(5) Scintigraphic evaluation of patients with chronic lung disease. Gallium scans have continued to play an important role in evaluation of chronic inflammatory disease. This technique is getting wider acceptance since its use was pioneered by the Pulmonary Branch in conjunction with the Department of Nuclear Medicine, Clinical Center. Gallium scans have been evaluated both in idiopathic pulmonary fibrosis and sarcoidosis in a prognosis use in determining which patients will deteriorate. Together with bronchoalveolar lavage this is a useful, nonevasive method to evaluate these patients. A new method has been developed with the Nuclear Medicine Department by which dynamic ventilation scans can be carried out both at rest and in exercise. These scans utilize ^{127}Xe in inhalation and then study the lung as a function of time. "Movies" can be made of the lung "breathing". Preliminary use of this method has suggested that it may be very useful in evaluating a variety of lung disorders such as destructive lung disease.

(6) Morphologic evaluation of fibrotic lung. Electron microscopic evaluation of upper lung biopsies from a large number of patients with interstitial lung disease have been evaluated. These biopsies have been gathered both at NIH and in collaboration with Dr. Francoise Basset, Hospital Bichat, University of Paris, through a cooperative agreement with INSERM and NIH. These studies have demonstrated a number of new findings in fibrotic lung including: (a) nuclear inclusions in epithelial cell nuclei; (b) accumulation of mast cells in the alveolar structures of patients with fibrotic lung disease; (c) a new structure, "anchoring fibrils", which are found in normal bronchi but not in alveolar structures and are present in large abundance beneath the basement membrane of patients with fibrotic lung disease; (d) evaluation of the derivation of the epithelial cells in fibrotic lung disease to demonstrate that they are not only derived from the existing epithelial cells but also from the bronchiolar epithelium; and (e) the evaluation of so-called "Langerhan's cells" in the lungs of patients with fibrotic lung disease, particularly those with histiocytosis-X.

(7) Physiologic studies. Evaluation of a number of sophisticated pulmonary function studies in conjuction with morphology of patients with idiopathic pulmonary fibrosis have demonstrated that exercise testing and volume-pressure relationships are the best physiologic methods to evaluate the actual structural abnormalities in these patients. Whereas changes in PaO_2 with exercise are secondary to both inflammatory and fibrotic changes, the alterations in volume-pressure relationships seem to be related only to the fibrosis. Detailed evaluation of Fabry's disease (Angiokeratoma Corporis Diffusion Universlae) a sex-linked sphingolipid disorder, has shown that these patients have airway disease. The functional abnormalities to limitation of the airflow is correlated with morphological findings of deposits of ceramide trihoxoside in epithelial cells of the bronchi of these patients. Functional evaluation of patients with Wegener's granulomatosis have demonstrated that they have functional abnormalities in volume, flow rates, and diffusing capacity. Whereas the first two seem to improve with therapy the diffusing capacity may continue to deteriorate. Functional parameters may be useful to follow these patients in addition to X-ray.

(8) Connective tissue changes in fibrotic lung disease. Evaluation of collagen concentration and rates of collagen synthesis in patients with idiopathic pulmonary fibrosis have demonstrated that both are normal. Although this may seem suprising and not consistent with the morphology, it suggests that the fibrotic process is much more complex than originally thought and likely involves alterations not only in the amount but also location, type and form of connective tissue. Evaluation of bronchoalveolar lavage of patients with fibrotic lung disease have shown that they have elevations of fibronectin, a connective tissue macromolecule known to mediate cell-cell and cell-matrix interactions. Normal individuals have the same levels of fibronectin in lavage fluid as in serum, suggesting that the elevation in the lungs of patients with fibrotic lung disease may be associated with the inflammatory process which destroys the fibronectin within the lung.

(9) Hereditary studies of connective tissue. Studies have continued to evaluate the lung function of patients with hereditary disorders of connective tissue such as osteogenesis imperfecta and Ehlers-Danlos syndrome (particularly type IV). Interestingly, most of the data to date suggest that lung function in these individuals is normal. Since it is known that each of these patients studied have marked abnormalities in connective tissue in other parts of the body, this would suggest that there may be more than one gene for each collagen type. For example, skin biopsies of patients with Ehlers-Danlos syndrome have a deficiency of type III collagen, if type III collagen is important in lung function (which it is thought to be) then it is unlikely that the same gene is operating in the lung parenchyma.

Significance to Biomedical Research and the Program of the Institute: The interstitial lung disorders are almost uniformly fatal and affect a significant proportion of the population. There has been little information on the natural history, etiology, pathogenesis, pathophysiology and therapy of these disorders. By combining studies of patients with these disorders with our basic research program concerning the control of synthesis and degradation of the extracellular matrix, we expect to make major inroads into understanding and treating these disorders.

Several of the studies outlined are long-terms studies but are of fundamental significance in the understanding of the structure and function of lung in health and disease. The connective tissue of lung is so complex that it would be difficult to determine the role of each component of the interstitial connective tissue unless methods such as those described above can be utilized. As more patients are evaluated, patterns should emerge which help to determine the contributions of each component of connective tissue as they relate to pulmonary structure and function.

Proposed Course: Studies as outlined will be continued. As methods are developed in the basic laboratory, they will be applied to study the biopsy specimens from human lung. Particularly important are the studies in lung explants and tissue culture where the manipulation and control of connective tissue synthesis and degradation can be explored using various pharmacologic agents. Immunologic studies will continue to explore cell-mediated mechanisms in these disorders. As the results of pharmacologic agents become promising, they will be studied in patients where applicable.

The α1-antitrypsin therapy project will be expanded to include study of heterozygotes. This is of particular importance because it has been suggested that patients heterozygous for the Z gene may be more at risk for the development of emphysema. Since Danazol is a relatively innocuous drug, it may bring the serum level of α1-antitrypsin above the threshold level by which these patients are more at risk.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 02409-05 PB

PERIOD COVERED
October 1, 1979 to September 30, 1980

TITLE OF PROJECT (80 characters or less)

Control of Synthesis and Degradation of the Extracellular Matrix

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	R. Crystal	Chief, Pulmonary Branch	NHLBI PB
Other:	G. Hunninghake	Staff Investigator	NHLBI PB
	J. Gadek	Staff Investigator	NHLBI PB
	K. Bradley	Chemist	NHLBI PB
	G. Fells	Biologist	NHLBI PB
	R. Zimmerman	Biologist	NHLBI PB
	B. Keogh	Staff Investigator	NHLBI PB
	C. Schoenberger	Clinical Associate	NHLBI PB
	P. Bitterman	Clinical Associate	NHLBI PB
	S. Rennard	Staff Investigator	NHLBI PB
	P. Tolstoshev	Expert	NHLBI PB
	S. Shibahara	Visiting Associate	NHLBI PB

COOPERATING UNITS (if any)

J. Moss	Staff Investigator	NHLBI CM
P. Kretschmer	Staff Investigator	NHLBI MH
B. Steinman	Staff Investigator	D LDBA

LAB/BRANCH
Pulmonary Branch

SECTION

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
15.9	8.5	7.4

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS ☒ (b) HUMAN TISSUES ☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The extracellular matrix of the alveolar structures is a critical determinant of lung structure and function in health and disease. Tissue culture studies have demonstrated a variety of lung cells both synthesize and destroy extracellular matrix complements. The fibroblasts synthesize collagen types I and III as well as producing a collagenase that destroys both collagen types. It also synthesized fibronectin, a glycoprotein important in cell-cell and cell-matrix interactions. The rate of collagen synthesis is rigidly controlled. However, there is environmental stimuli such as prostaglandins and mediators of activated lymphocytes can alter the differentiated state of fibroblasts with respect to collagen synthesis. The intracellular degradation of collagen has been defined: approximately one-third of all collagen synthesized by fibroblasts is destroyed within the cell prior to secretion. The portion of intracellular degradation can be modulated by altering the structure of collagen or by changing the levels of intracellular cyclic AMP. Studies of collagen gene structure have shown that the pro α2 gene contains significant numbers of noncoding sequences.

Other:	C. Boyd	Guest Worker	NHLBI PB
	R. Haber	Bio Lab Tech	NHLBI PB
	B. Trapnell	Biological Aid	NHLBI PB
	M. Schafer	Chemist	NHLBI PB
	J. Davidson	Staff Investigator	NHLBI PB
	K. Smith	Biologist	NHLBI PB
	R. Dalgleish	Guest Worker	NHLBI PB
	M. Schwartz	Chemist	NHLBI PB
	Larue Stier	Chemist	NHLBI PB
	L. Saltzman	Biologist	NHLBI PB
	R. Berg	Staff Investigator	NHLBI PB

Cooperating Units:

G. Martin	Chief, Laboratory of Developmental Biology and Anomalies	D LDBA

Project Description:

Objectives: Current concepts of the extracellular matrix of the alveolar structures suggest it is composed of four general classes of materials: (1) collagen, of which there are four types (types I and III are the interstitial collagens, types IV and V are the principle basement membrance collagens); (2) elastic fibers; (3) proteoglycans; and (4) fibronectin, a newly described class of glycoproteins involved in cell-cell and cell-matrix interactions. In the past year, our studies have concentrated on the control of connective tissue production and destruction.

Major Findings: Over the past year the major findings in this area have been as follows:

(1) Regulation of collagen production by human fibroblasts. Human lung fibroblasts have been studied in detail concerning their regulation of collagen production. Normally these cells maintain very tight control over the amounts of collagen they produce. For example, collagen production during periods of rapid growth occurs at an identical rate as that during slow growth. In addition, fibroblasts of early passage produce the same amounts of collagen as do fibroblasts that have gone through several population doublings. However, even though these cells tightly regulate the amounts of collagen they can produce, they can modulate their collagen production. One important mechanism by which they do this is through intracellular degradation. Fibroblasts have mechanisms to "shunt" collagen from the normal secretory pathway to the lysosomal system. Detailed evaluation of this process has suggested that at least two "signals" can induce fibroblasts to increase intracellular collagen degradation. First, if the cell produces non-helical collagen, the intracellular degradation mechanisms recognize this and destroy this structurally abnormal collagen before it is secreted. Second, any signal that increases intracellular cyclic AMP will result in an increase in intracellular collagen degradation. Over the past year, methods have also been developed to quantitate messenger RNA levels and activities for type I collagen synthesis in cultured fibroblasts. Studies of cells during periods of rapid and slow growth have shown that there are significant alterations in mRNA levels although the activity of the mRNA (per molecule) is similar.

(2) Evaluation of cyclic AMP levels in human lung fibroblasts has demonstrated that this cyclic nucleotide is critically linked to collagen production. Agents that increase intracellular cyclic AMP (e.g., isoproternol, PGE_1, cholera toxin) all decrease collagen production by these fibroblasts. Other β-stimulents such as norepinephrine and epinephrine also caused this phenomena. The concept has been developed that under normal conditions fibroblasts of the body are generally "suppressed" in terms of their potential

for collagen production by endogenous β-stimulants. This suggests that the β-adrenergic system "holds back" collagen production and thus has the potential for significantly modifying the quantity of extracellular matrix components. This also leads to possible mechanism of β-agonist-induced fibrosis, i.e., when propanolol is given to a susceptible individual, this β-blocker prevents the normal suppression of collagen production by endogenous β-agonists and thus increases the produciton of collagen in certain organs.

(3) Evaluation of collagen gene structure. In the past year, a major advance has been made in the evaluation of collagen gene structure through the isolation of a collagen gene using the methodologies of recombinant DNA. This collagen gene represents approximately 50% of the sheep pro α2 gene. It is one of the most complex and largest genes isolated to date. The structural information for 50% of the pro α2 mRNA is contained in 2.5 kilobases of DNA whereas 50% of the gene itself is approximately 17 kilobases. The vast majority of this gene is comprised of "introns" with only a small portion representing "exons" (the structural information). Evaluation of the structure of the sheep pro α2 gene using restriction enzymes and electron microscopic R loop mapping have shown that the exons are interspersed among the very large introns. Other studies from the laboratory have theoretically evaluated these so-called "splice-junction structures" of a variety of genes. These structures are the regions between exons and introns and seem to be represented by "consensus sequences", i.e., similar sequences within the same gene and among different genes where the exons are "cleaved out" to be subsequently rejoined to the final messenger RNA. Theoretical evaluation of these splice-junction structures have shown that for all known mRNA precursors, the regions around the splice junctions form stable secondary RNA structures in which the "consensus" sequences are "available" for splicing.

(4) Studies of collagen production in tendon, skin, and lung of sheep of varying gestational age have demonstrated that messenger RNA levels significantly control the production of collagen by these tissues. The percentage of protein synthesis devoted to collagen production varies in these tissues as a function of gestational age during fetal development, and appears to be paralleled, in general, by collagen messenger RNA levels. The exception is in skin, where high messenger RNA levels are maintained late in gestational life whereas the tissue appears not to be using all the messenger RNA that is present.

(5) Studies of elastin production in developing ligamentum nuchae and lung. Methods have been developed to quantitate the amount of elastin in various tissues as well as the rate of elastin synthesis by small pieces of tissue maintained in short term incubations. The technology has been developed to isolate and quantitate the elastin messenger RNA. Studies of these devel-

oping systems have shown that, in general, the rate of elastin production seems to be controlled by the amounts of available elastin mRNA in the tissue. In addition, elastin mRNA has been purified and a complementary DNA made to it. This cDNA is being used to probe tissues and cells for amounts of messenger RNA and to screen genomic "libraries" in order to isolate the elastin gene.

(6) Evaluation of hereditary disorders of connective tissue. Osteogenesis imperfecta is a disease associated with brittle bones and is thought to represent an abnormality of type I collagen. One hypothesis to explain this disease is that there is a single base substitution in the gene structure resulting in an amino acid substitution that yields instability of the collagen helix. To evaluate the hypothesis that such collagen would be degraded within the cells prior to secretion, fibroblasts from a variety of patients with osteogenesis imperfecta were compared for their quantity of intracellular collagen degradation compared to control fibroblasts. Although the studies have shown no difference, this may result from the fact that the fibroblasts used were derived from skin whereas the disease primarily affects bone. As such, this may imply there is more than one type I collagen gene (if the defect is within the type I molecule). Studies are in progress concerning the Ehlers-Danlos IV syndrome, an hereditary disorder of connective tissue in which type III collagen is not made. Messenger RNA for collagen can be isolated from fibroblasts from these patients and are being compared to normal fibroblasts. To search for the underlying genetic defect in this disorder, studies are utilizing these fibroblasts to isolate messenger RNA for collagen types I and III and to evaluate type I and type III collagen gene structure.

(7) Destruction of connective tissue components. Collagenase is an enzyme which specifically cleaves the collagen molecule at a point three-quarters of the distance from the N-terminal end. It has been thought that collagenase is the only neutral protease that has access to connective tissue that will attack collagen in the "collagenase" sensitive site. However, evaluation of human neutrophil elastase has shown that it can also act as a "collagenase" even though it has a broad spectrum of other proteolytic activities. Human neutrophil elastase will cleave type III collagen at a site close to the "collagenase" site. However, elastase does not attack type I collagen. In contrast, human neutrophil collagenase attacks type I collagen but not type III collagen. Thus, we must expand our concepts of collagenases, elastases and other proteolytic enzymes to include the concept that they may have a broader spectrum of activity than previously thought.

Significance to Biomedical Research and the Program of the Institute: The extracellular matrix of the alveolar structures is a critical determinant of lung structure and function. Mechanical properties of lung are intimately involved with the type, form, location and orientation of extracellular matrix.

In addition, the topologic arrangement of cells is defined in part by the macromolecules of this matrix. It is likely that the connective tissue of the interstitium also forms a line of defense against inhaled agents and control in part the movement of solutes and water from the capillaries to the alveolar structures. The pathogenesis of a large number of pulmonary diseases afflicting mankind are intimately involved with abnormalities in the connective tissue. The emphysematous lung disorders are involved in destructive processes of the extracellular matrix and the interstitial lung disorders are involved primarily with abnormalities in the collagen of the extracellular matrix. Understanding of the control of synthesis and degradation of these materials is of obvious importance in terms of understanding the pathogenesis of these disorders as well as understanding vulnerable points of attack for therapeutic intervention of these disorders.

Proposed Course: Our studies will continue to evaluate the composition and source of the macromolecules composing the extracellular matrix. In addition, detailed studies will continue on the control of the synthesis and degradation of these macromolecules at the cellular and sub-cellular levels.

Publications:

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ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG AND BLOOD INSTITUTE
October 1, 1979 through September 30, 1980

Monoclonal Antibodies As Probes For Neural or Glial Molecules and Functions. Spleen cells from mice immunized with cells from dorsal or ventral 14-day chick embryo retina were fused with clonal mouse myeloma cells to obtain monoclonal antibodies with topographic specificity for neural retina. One of 177 hybridoma cell lines examined was found to synthesize antibody directed against cell membrane molecules distributed in a dorsal → ventral concentration gradient in retina. The concentration of antigen is at least 35-fold higher at the dorso-posterior margin of the retina than at the ventro-anterior margin, and the antigen concentration is a logarithmic function of distance between the dorsal and ventral poles of the gradient along the circumference of the retina. Bilaterally symmetrical, mirror-image gradients of antigen were found in right and left retina. A gradient of antigen was detected in retina at each stage of embryonic development tested (4 thru 18 day embryos) and in adult retina. The antigen was found, in order of decreasing concentration, in retina, cerebrum, thalamus, optic nerve, cerebellum, and optic tectum; in contrast, little or no antigen was found in heart, liver, kidney, or blood cells. Antigen gradients were found in chicken, quail, duck and turkey retina, but not in goldfish or rat retina.

Immunofluorescence and autoradiography revealed the antigen on the surface of most, or all, cells and cell types in dorsal chick embryo retina. Thus, the antigen gradient can be used as a marker for the dorsoposterior-ventroanterior axis of the retina and for cell position in retina along this axis. Further work is needed to determine whether the antigen plays a role in the coding of positional information in the retina and the specification of synaptic connections.

Forty-six additional hybridoma cell lines that synthesize antibodies directed against retina antigens also were obtained. One antibody is specific for neurons; another for retina Müller cells; and a third antibody recognizes surface molecules of retina cells that decrease in concentration during the course of development.

Mice also were immunized with clonal neuroblastoma x retina hybrid cells, fibroblast x retina hybrid cells, or synaptosome membrane preparations. Examination of more than 1500 hybridomas yielded more than 200 cell lines that synthesize antibodies directed against cells or membranes used for immunization. Several antibodies were found that increase the frequency of miniature end-plate potentials at synapses between neuroblastoma-hybrid cells and striated muscle cells. Other antibodies exhibit specificity for synaptosome membrane antigens, or for regions within the nervous system. Antibodies that recognize developmentally regulated antigens also were detected. Preliminary results suggest that two monoclonal antibodies have been obtained that recognize receptors for neurotransmitters. Further studies on antibody characterization and antigen purification are in progress.

Biochemistry of Synaptogenesis. Synapses between clonal neuroblastoma hybrid cells and striated muscle cells exhibit a high degree of plasticity. Activation of adenylate cyclase by PGE_1 or treatment of cells with dibutyryl cyclic-AMP gradually results in the appearance of functional, voltage-sensitive Ca^{2+} channels. A depolarizing stimulus then results in activation of voltage-sensitive calcium channels, influx of Ca^{2+} ions, and secretion of acetylcholine by cells. Thus, reactions that regulate the acquisition of voltage-sensitive Ca^{2+} channels, in turn, control synapse plasticity in this model system.

Ca^{2+} fluxes resulting from activation of NBr10-A voltage-sensitive calcium channels were assayed with a calcium-specific electrode, by a spectrophotometric assay based on the formation of ($Ca^{2+}\cdot$murexide) complexes using a stopped-flow apparatus and a dual wavelength spectrophotometer, by measuring $^{45}Ca^{2+}$ fluxes, and by recording intracellular membrane potential. The results obtained by the different methods agree well. In addition, the results obtained by the spectrophotometric and the Ca^{2+} specific electrode methods show that activation of voltage-sensitive calcium channels results in net uptake of Ca^{2+} ions by NBr10-A cells, which is followed by a slow compensatory efflux of Ca^{2+} until a new equilibrium between internal and external Ca^{2+} ions is achieved.

Most of the Ca^{2+} ions within NBr10-A cells are sequestered in mitochondria but an ATP-dependent Ca^{2+} uptake by smooth endoplasmic reticulum or microsome fractions from NBr10-A was demonstrated that is insensitive to inhibitors of mitochondrial Ca^{2+} uptake.

A neuroblastoma x glioma hybrid cell line was found that lacks nicotinic acetylcholine receptor aggregating protein. The hybrid cells do not form synapses with striated muscle cells because nicotinic acetylcholine receptors do not aggregate on myotube membranes at points of contact with the hybrid cells.

Neuroblastoma N1E-115 cells were found to possess two types of K^+-channels. One channel is activated by cell depolarization; activation of the other K^+-channel is dependent on Ca^{2+} ions and is not voltage-sensitive. The Ca^{2+}-activated K^+-channel is inhibited by 50 μM quinine, thus decreasing after-hyperpolarization and slow oscillations in the membrane potential of the cells. The Ca^{2+}-dependent activation of K^+-channels regulates the ability of N1E-115 cells to generate repetitive action potentials in response to a stimulus.

Previously, NG108-15 neuroblastoma x glioma cells were shown to release a macromolecule into the medium that induces localized aggregation of nicotinic acetylcholine receptors on striated muscle cells. The receptor aggregating protein is released into the medium by cultured neurons that synthesize acetylcholine and also by neurons that do not synthesize acetylcholine. The receptor aggregation factor was partially purified and was shown to be a protein. Factor-dependent aggregation of nicotinic acetylcholine receptors on muscle plasma membranes is dependent on Ca^{2+} ions, is responsive to temperature, and is inhibited by sodium azide or by colchicine.

These results suggest that aggregation of acetylcholine receptors is a Ca^{2+} and energy-dependent process that may involve microtubules. The rate of extraction of nicotinic acetylcholine receptors from myotube membranes by detergents was found to decrease with maturation of the myotubes; concomitantly, the density of the detergent resistant cytoskeletal layer beneath the plasma membrane increases.

Two classes of endogenous opioid peptides have been identified: enkephalin pentapeptides, and higher molecular weight endorphin peptides. Affinity-purified antibodies reactive against the carboxyl-terminal determinants of Met-enkephalin and Leu-enkephalin were prepared and used to study enkephalin biosynthesis. The antibodies were shown to interact with 5 species of peptides or protein from adrenal medulla with molecular weights of 570, 1,100, 18,000, 26,000, and 40,000. Treatment of the protein with a molecular weight of 40,000 with trypsin and carboxypeptidase B yielded enkephalins.

Messenger RNA was isolated from adrenal medulla and used to direct the cell-free synthesis of protein labeled with [^{35}S]-methionine. Treatment of the ^{35}S-protein with trypsin and carboxypeptidase B yielded an ^{35}S-peptide that was purified by immunoprecipitation and high-pressure liquid chromatography and identified as [^{35}S]Met-enkephalin. These results show that mRNA from adrenal medulla can direct the cell-free synthesis of a Met-enkephalin precursor protein and provides a system that should be useful for studies on enkephalin biosynthesis and for the synthesis and cloning of a gene for the enkephalin precursor protein.

To devise molecular probes and affinity labels for α-adrenergic receptors, several analogs of clonidine, an α_2-adrenergic receptor partial agonist, and other α-adrenergic ligands were synthesized and tested for binding to α-receptors of rat brain and NG108-15 neuroblastoma x glioma hybrid cells.

Clonidine, p-aminoclonidine, and N-(4-hydroxyphenacetyl)-4-aminoclonidine (HP-aminoclonidine) were shown to be potent partial agonists for α_2-receptor mediated inhibition of NG108-15 adenylate cyclase (K_{Dapp} 300, 50, and 130 nM, respectively). These compounds also bind with high affinity to α_2-receptors of rat brain and NG108-15 membranes as determined by displacement of bound [^{3}H]clonidine. [^{3}H]Clonidine binds to NG108-15 membranes receptors with K_{Dapp} values of 1.7 and 33 nM for high- and low-affinity sites, respectively. Classical α-adrenergic agonists and antagonists displace [^{3}H]clonidine from sites. p-Aminoclonidine and HP-aminoclonidine displace [^{3}H]clonidine from the high-affinity sites with K_D values of 2.3 and 5.8 nM, respectively.

The affinities of the ligands for NG108-15 α-receptor binding sites were higher than the corresponding potencies for inhibition of NG108-15 adenylate cyclase. This discrepancy can be explained by the demonstration that GTP, ATP, Na$^+$ ions inhibit binding of α-receptor agonists to the high affinity site. GTP and probably Na$^+$ ions are required for α-receptor-mediated inhibition of adenylate cyclase; thus, the high-affinity [^{3}H]clonidine sites may represent uncoupled receptors, while the low affinity sites may represent receptors coupled to adenylate cyclase.

HP-Aminoclonidine possesses opiate receptor agonist activity in addition to α-receptor partial agonist activity. This analog inhibits NG108-15 adenylate cyclase (K_D 0.7 μM), and most of the inhibition is reversed by opiate receptor antagonists. HP-Aminoclonidine also displaces [^{3}H]dihydromorphine and [^{3}H]D-Ala-Met-enkephalinamide from rat brain opiate receptors with IC_{50} values of 0.6 and 0.12 μM, respectively.

Other compounds synthesized as potential affinity labels for α_2-receptors include bromo-acetyl-and fluorosulfonyl-derivates of aminoclonidine, yohimbine, and phentolamine. Several promising compounds will be studied further.

Exposure of E. coli cells to sugars and certain other carbon sources results in a decrease in cyclic AMP levels of cells. We previously showed that glucose transport into cells results in an inhibition of adenylate cyclase. An analysis of the properties of strains of E. coli harboring mutations in the sugar transport system energized by phosphoenolpyruvate (abbreviated PTS) has revealed that PTS activity influences adenylate cyclase activity. PTS proteins play a role as phosphate carriers and can, therefore, exist in either a phosphorylated or dephosphorylated state. As a result of our observation that some mutants in the PTS protein, Enzyme I, have reduced levels of adenylate cyclase activity, which can, under certain conditions, be stimulated by phosphoenolpyruvate, we have proposed a scheme for regulation of adenylate cyclase activity by the PTS. According to this scheme, Enzyme I interacts with the catalytic unit of adenylate cyclase. When there is an abundance of cellular phosphoenolpyruvate and a deficiency of extracellular glucose, the PTS proteins are largely phosphorylated; a high level of phosphorylation of PTS proteins is associated with a high level of adenylate cyclase activity. On the other hand, when the cells are exposed to a high concentration of extracellular sugar, the PTS effects the transport of the sugar resulting in the accumulation of intracellular sugar phosphate. Accompanying the sugar transport, there is a concomitant dephosphorylation of the PTS proteins; a low level of phosphorylation of the PTS proteins is associated with a low level of adenylate cyclase activity.

We previously showed that histidyl-proline diketopiperazine is a metabolite of thyrotropin releasing hormone and that the peptide is more active than thyrotropin releasing hormone in antagonizing the sleep-inducing effects of ethanol in rats. Injection of either thyrotropin releasing hormone or histidyl-proline diketopiperazine results in elevation of cyclic GMP levels in the brain. However, antagonistic effects of hormone and metabolite on body temperature were observed. During the past year, histidyl-proline diketopiperazine was shown to be a noncompetative inhibitor of dopamine uptake into synaptosomes. Exploration of the mechanism of inhibition of catacholamine transport by the peptide revealed that histidyl-proline diketopiperazine inhibits sodium-potassium dependent ATPase that generates an ion gradient necessary for catecholamine uptake. The portion of the ATPase that interacts with the cell interior and is inhibited by vanadate is modified by histidyl-proline diketopiperazine.

1076

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00009-06 LBG

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Cell Recognition and Synapse Formation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Marshall Nirenberg	Chief, LBG	LBG, NHLBI
OTHER:	Michael Adler	Staff Fellow	LBG, NHLBI
	Neil Busis	Medical Officer	LBG, NHLBI
	Paul Darveniza	Visiting Associate	LBG, NHLBI
	Angel DeBlas	Visiting Fellow	LBG, NHLBI
	Joseph Moskal	Staff Fellow	LBG, NHLBI
	Radharaman Ray	Staff Fellow	LBG, NHLBI
	Michael Schneider	Medical Officer	LBG, NHLBI
	David Trisler	Staff Fellow	LBG, NHLBI

COOPERATING UNITS (if any)

None

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section of Molecular Biology

INSTITUTE AND LOCATION
NIH, NHLBI, Bethesda, Maryland 20205

TOTAL MANYEARS: 11	PROFESSIONAL: 9	OTHER: 2

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SUMMARY OF WORK (200 words or less - underline keywords)

Presynaptic reactions were identified which regulate the number of NBr10-A synapses with striated muscle cells and the efficiency of trans-synaptic communication. Synapse plasticity was regulated by the acquisition of functional voltage-sensitive Ca^{2+} channels by NBr10-A cells.

Monoclonal antibodies were used as probes to detect molecules needed for synaptogenesis or transynaptic communication. Antibodies were obtained which either affect the frequency of miniature end-plate potentials at synapses, recognize certain cell types in the nervous system, or exhibit regional specificity in the nervous system. An antibody with topographic specificity also was obtained which recognizes cell surface molecules that are distributed in a dorsal → ventral concentration gradient in avian retina. The gradient of antigen can be used to define the dorsoposterior → ventroanterior axis of the retina and the relative positions of cells in retina along this axis.

Project Description:

Major Findings:

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Significance to Biomedical Research:

Little is known about the biochemical basis for synapse plasticity. The demonstration that the presynaptic acquisition of voltage-sensitive Ca^{2+} channels can be regulated and that this process can turn synapses on or off is of basic interest with respect to the development of the nervous system and memory.

The identification of molecules distributed in retina in a spatially organized pattern raises the possibility that the molecules may play a role in the coding of positional information in retina and the specification of synaptic connections.

Proposed Course:

Further work is needed to clarify antibody specificity, to determine the effects of antibodies on neural or glial functions, and to characterize and purify certain antigens.

Publications:

1. Eisenbarth, G.S., Walsh, F.S. and Nirenberg, M. Monoclonal antibody to a plasma membrane antigen of neurons. Proc. Natl. Acad. Sci. USA, 76, 4913-4917 (1979).

2. Nirenberg, M., Wilson, S., Higashida, H., Thompson, J., Eisenbarth, G., Walsh, F., Rotter, A., Kenimer, J. and Sabol. S. Synapse Plasticity. In Pontificiae Academiae Scientarvm Scripta Varia, in Press.

3. Schneider, M.D. and Eisenbarth, G.S. Transfer plate radioassay using cell monolayers to detect anti-cell surface antibodies synthesized by lymphocyte hybridomas. J. Immunol. Methods, 29, 311-342 (1980).

4. McGee, R., Smith, C., Christian, C., Mata, M., Nelson, P. and Nirenberg, M. A new methods for measurement of the uptake and release of materials from cultured cells. Analytical Biochem., 101, 320-326 (1980).

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6. Wilkening, D., Sabol, S.L. and Nirenberg, M. Control of opiate receptor-adenylate cyclase interactions by calcium ions and guanosine 5'-triphosphate. Brain Research, 189, 459-466 (1980).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00017-05 LBG

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October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Acetylcholine Receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Mathew P. Daniels	Research Biologist	LBG NHLBI
OTHERS:	C. Christian	Senior Staff Fellow	LDN NICHD
	Hans Bauer	Visiting Scientist	LDN NICHD
	Joan Prives	Guest Worker	LDN NICHD
	Anne Schaffner	Guest Worker	LBG NHLBI

COOPERATING UNITS (if any)
Laboratory of Developmental Neurobiology, NICHD
Neurobiology Unit, Weizmann Institute of Science

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, MD 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
6.0	4.0	2.0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Our aim has been to reveal the topographic distribution of neurotransmitter receptors on nerve and muscle cells, while relating this distribution to the development and function of synapses. To this purpose, we have used α-bungarotoxin as a specific probe for the visualization and quantitation of nicotinic acetylcholine receptor sites. Our recent work has emphasized a model system for studying the neural control of nicotinic acetylcholine receptor distribution on skeletal muscle fibers. This system is based on the finding that embryonic and clonal nerve cells produce a factor which induces aggregation of receptors on cultured skeletal myotubes. We have now provided evidence that: 1) the factor is a large protein; 2) it activates an energy-dependent mechanism in the myotube, probably involving cytoskeletal components; 3) a similar factor, or factors, are produced by non-cholinergic as well as cholinergic nerve cell types.

Project Description:

Objectives:

Our aim has been to reveal the topographic distribution of neurotransmitter receptors on nerve and muscle cells, while relating this distribution to the development and function of synapses. Our recent work has focussed on the extrinsic and intrinsic influences on the distribution of nicotinic acetylcholine receptors (AChR) on the surface of the developing muscle fiber. In particular, we have been investigating the mechanisms whereby soluble, macromolecular factors from neurons can induce localized aggregation of AChR as occurs in the developing neuromuscular synapse.

Methods:

We have stained skeletal myotubes grown in monolayer culture with rhodamine-labeled α-bungarotoxin (αBT) in order to visualize and quantitate AChR sites with the fluorescence microscope. AChR aggregating factor has been prepared from culture medium conditioned by primary embryonic neurons or clonal nerve cell lines; or from high-speed supernatents of homogenized nerve cells and tissues.

^{125}I-labeled αBT binding, gentle detergent treatment, and electron microscopy have been used to study AChR-cytoskeletal interactions.

Major Findings:

Protease digestion demonstrated that the AChR aggregation factor is a protein.

The mechanism of action of the AChR aggregation factor from neuroblastoma x glioma hybrid cells (NG108-15) was further investigated by manipulating the environment of muscle cultures during a 4-6 hour treatment with concentrated conditioned medium (CM) from NG108-15 cell cultures. The CM-induced aggregation of AChR on the muscle cells was inhibited by low temperature, sodium azide, removal of Ca^{++} from the medium and by colchicine. Cytochalasin B had no effect. These results suggest that the CM-induced aggregation of AChR is an energy and Ca^{++} dependent process, possibly involving microtubules.

Further studies utilizing gentle detergent extraction of muscle cultures indicated that the average rate of detergent extraction of AChR decreases with maturation of the cultures, in conjunction with an increase in the overall density of the detergent resistant cytoskeletal layer beneath the plasma membrane. This is consistant with the postulated interaction between the receptors and cytoskeleton.

Release of high molecular weight AChR aggregating activity and acetylcholine synthesis were studied in pure cultures of neurons from embryonic or newborn superior cervical ganglion, spinal cord, cerebellum, and dorsal root ganglion. No correlation was found between AChR aggregating activity and synthesis of acetylcholine. It is possible that production of neurotransmitter receptor aggregating factors is a general property of developing neurons.

Significance to Biomedical Research:

Knowledge of the topographical distribution of neurotransmitter receptors on nerve and muscle cells is of clear importance in any attempt to understand the role of neurotransmitters and their receptors in the function and development of the nervous system. Our studies on the AChR aggregation factor may lead to a better understanding of the mechanisms whereby neurons control or modulate the distribution of receptors on muscle fibers and on other neurons, during synapse development and after.

Proposed Course:

1) Detailed light - and electronmicroscopic analysis of AChR aggregate formation and the underlying structural mechanisms has been initiated.

2) An "immunochemical" assay for receptor aggregation activity, utilizing gentle detergent extraction of myotube cultures after binding specific antibody to the receptors, is under development. This assay will be many times faster than the present microscopic assay, and should facilitate our efforts to further purify the factor and determine its mechanism of action.

3) Since AChR aggregation factor has been detected in CM from a variety of embryonic neuron types, including at least two that are not cholinergic, we will try to determine whether the same or similar factors can induce aggregation of other neurotransmitter receptors, such as GABA or glutamate receptors on cultured spinal cord neurons.

Publications:

1) Daniels, M.P. and Vogel, Z.: Localization of α-bungarotoxin binding sites in synapses of the developing chick retina. Brain Res. In Press, 1980.

2) Christian, C.N., Bergey, G.K., Daniels, M.P. and Nelson, P.G.: Cell interactions in nerve and muscle cell cultures. Woods Hole Workshop on Neurotransmission, Neurotransmitters and Neuromodulators. J. Exp. Biol. In Press, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00018-03 LBG

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)
Regulation of the bio-synthesis and secretion of opioid peptides
(Former Title: Regulation of synthesis and secretion of endorphins by pituitary tumor cells.)

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Steven L. Sabol	Medical Officer	LBG NHLBI
OTHERS:	Satyaprabha Dandekar	Visiting Fellow	LBG NHLBI

COOPERATING UNITS (if any)
None.

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Laboratory of Biochemical Genetics

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INSTITUTE AND LOCATION
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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Methionine-enkephalin and leucine-enkephalin are endogenous pentapeptides that possess opiate activity. The bovine adrenal medulla, which synthesizes and secretes enkephalins, has been chosen for the investigation of the biosynthesis of the enkephalins. Messenger RNA (mRNA) from adrenal medulla has been isolated and used as a template for the cell-free synthesis of enkephalin precursor protein in the wheat germ and rabbit reticulocyte protein-synthesis systems. Specific proteolytic digestion of the adrenal mRNA-dependent proteins yields methionine-enkephalin. The enkephalin precursor protein and enkephalin mRNA are currently being characterized with the goal of understanding the regulation of synthesis of enkephalins, which are thought to be important in pain perception and behavior.

Project Description:

Objectives:

Two systems of endogenous opioid peptides have been distinguished: (1) the β-endorphin system found in the anterior pituitary and hypothalamus, and (2) the enkephalin system found in the brain, intestinal tract, spinal cord, adrenal medulla, and elsewhere. Previous work on this project dealt with the β-endorphin system, in particular on the regulation of synthesis and secretion of β-endorphin and its biosynthetic precursors in clonal AtT-20 mouse pituitary tumor cells. This work is summarized in the Annual Report of last year. While knowledge now exists concerning the biosynthesis of β-endorphin, little is known about the biosynthesis of the enkephalins. Larger peptides containing enkephalins have recently been isolated from bovine adrenal medulla and are being characterized by others. We have adopted the alternative approach of in vitro translation of enkephalin precursor mRNA to provide information concerning the earliest and largest putative enkephalin precursor ("pre-pro-enkephalin"), the mRNA coding for this precursor, the gene(s) for pre-pro-enkephalin, and the regulation of expression of the enkephalin gene(s).

Methods Employed:

Discussed in Major Findings below.

Major Findings:

Antisera reactive against the carboxyl-terminal determinants of Met-enkephalin and Leu-enkephalin were prepared and radioimmunoassays for the enkephalins were set up in order to determine the reactivity of the antisera with proteins in the adrenal medulla extracts. The antisera, as well as affinity purified antibodies derived from them, react not only with enkephalins (molecular weight 570) but also with peptides of molecular weight approximately 11,000, 18,000, and 26,000. An even larger peptide (molecular weight 40,000) that yields enkephalins upon trypsin-carboxypeptidase B digestion, is very weakly reactive. We conclude from the specificity of the antisera that the bovine adrenal possesses probably precursor proteins that contain enkephalin residues at their carboxyl-terminal ends.

Adrenal medulla mRNA, when translated in cell-free systems, directs the incorporation of [^{35}S]methionine into many proteins as analyzed by polyacrylamide gel electrophoresis. None of these synthesized proteins appears to be specifically recognized by the enkephalin antisera, but after these proteins are treated with trypsin and then carboxypeptidase B, small amounts of [^{35}S]Met-enkephalin can be detected by immunoprecipitation and analysis of the immunoprecipitated peptides by high-pressure liquid chromatography. The characterization of the protein(s) yielding enkephalins is in progress.

Significance to Biomedical Research:

The preliminary results obtained so far suggest that we will be able to identify and characterize the pre-pro-enkephalin biosynthetic precursor, and to subsequently study the molecular genetics of the enkephalin system. This information should be of great value in understanding the physiological role of enkephalins, which act as neurotransmitters or neuromodulators in the nervous system and which may also act in a hormonal manner when released into the blood by the adrenal medulla during stress. Systems to study the biosynthesis of enkephalins will be useful to evaluate the theory that chronic opiate dependence involves a reduced biosynthesis of enkephalin.

Proposed Course:

1. Peptide mapping of the pre-pro-enkephalin synthesized in vitro and determination of its ability to be glycosylated.

2. Purification of enkephalin mRNA using a custom-synthesized dodecadeoxynucleotide as a probe that will specifically hybridize with the region of the mRNA coding for the sequence of Met-enkephalin$_{2-5}$.

3. Synthesis of cDNA complementary to enkephalin mRNA using the same synthetic dodecadeoxynucleotide as a primer for reverse transcriptase, then sequencing of the cDNA. The cDNA may be cloned in an *E. coli* phage system.

4. Studies on the regulation of enkephalin transcription.

5. Preparation of antisera or monoclonal antibodies against the precursor to allow direct immunoprecipitation.

Publications:

1. Sabol, S.L.: Storage and secretion of β-endorphin and related peptides by mouse pituitary tumor cells: regulation by glucocorticoids. Arch. Biochem. Biophys. 203, 37-48, (1980).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00151-10 LBG

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

The Biology of Cyclic Nucleotides in *E. coli*

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Peterkofsky	Research Chemist Chief, Section on Macromolecules	LBG, NHLBI
OTHER:	Robert Puskas	Staff Fellow	LBG, NHLBI

COOPERATING UNITS (if any)

None.

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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2.5	1.5	1

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

This project involves a continuing effort to develop an understanding of the role of cyclic nucleotides (cyclic AMP and cyclic GMP) in Escherichia coli and the mechanism by which the levels of these nucleotides is regulated within the cells under a variety of conditions. Our findings this year have dealt with the regulation of the activity of adenylate cyclase by the phosphoenolpyruvate linked phosphotransferase system for transport of sugars (PTS). Mutants in one of the proteins of this transport system (Enzyme I) lead to a depression of cellular cyclic AMP levels. Under appropriate conditions, the levels of cyclic AMP in these mutants can be restored to normal by exposure to the phosphorylating agent, phosphoenolpyruvate. These data have been interpreted in the framework of a model for regulating adenylate cyclase activity by interaction with proteins of the PTS, such that the state of phosphorylation of the PTS proteins determines the activity of the enzyme.

PHS-6040
(Rev. 10-76)

Major Findings:

Our efforts during the past nine years have been directed at developing an understanding of the mechanism by which the levels of cyclic AMP in E. coli are lowered when the cells are exposed to carbon sources, generally sugars. We have been able to demonstrate that the locus of action of sugars involves an inhibition of the synthesis of cyclic AMP and that, in the case of glucose, some aspect of the transport of the sugar into the cells results in an inhibition of adenylate cyclase activity. An analysis of the properties of strains of E. coli harboring mutations in the sugar transport system energized by phosphoenolpyruvate (abbreviated PTS) has revealed that the PTS activity influences adenylate cyclase activity. PTS proteins play a role as phosphate carriers and can therefore exist in either a phosphorylated or dephosphorylated state. As a result of our observation that some mutants in the PTS protein known as Enzyme I have reduced levels of adenylate cyclase activity, which can, under certain conditions, be stimulated by phosphoenolpyruvate, we have proposed a scheme for regulation of adenylate cyclase activity by the PTS. According to this scheme, the PTS protein called Enzyme I interacts with the catalytic unit of adenylate cyclase. When there is an abundance of cellular phosphoenolpyruvate and a deficiency of extracellular glucose, the PTS proteins are phosphorylated to a large degree; a high level of phosphorylation of PTS proteins is associated with a high level of adenylate cyclase activity. On the other hand, when the cells are exposed to a high concentration of extracellular sugar, the PTS effects the transport of the sugar resulting in the accumulation of intracellular sugar phosphate. Accompanying the sugar transport, there is a concomitant dephosphorylation of the PTS proteins; a low level of phosphorylation of the PTS proteins is associated with a low level of adenylate cyclase activity.

Proposed Course of Research:

The results of our studies on the mechanism by which the PTS regulates the activity of adenylate cyclase has led to the development of the concept that a phosphorylation-dephosphorylation mechanism controls the activity of adenylate cyclase. This idea has been accepted in the literature. However, studies of other investigators have raised questions as to the identity of the specific protein of the PTS which actually complexes with the catalytic unit of adenylate cyclase. The scheme we have developed proposes that Enzyme I is the regulator protein, while another group has suggested a different protein as the candidate for the regulator. Some of our efforts in the future will be directed to attempts to resolve this question. We will make attempts to purify adenylate cyclase as well as various PTS proteins and then try to reconstitute complexes that show regulatory properties. At the same time, we will continue to learn more about the factors other than the PTS that play a role in regulating the activity of adenylate cyclase.

Publications:

1) Peterkofsky, A. and Gazdar, C.: The Escherichia coli adenylate cyclase complex: activation by phosphoenolpyruvate. In: Bitensky, M., Collier, R.J., Steiner, D.F. and Fox, C.F. (eds.). Progress in Clinical and Biological Research, Vol. 31: Transmembrane Signalling. Alan R. Liss, New York, pp. 233-244, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00152-06 LBG

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Metabolism of Peptide Hormones

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Alan Peterkofsky	Research Chemist Chief, Section on Macromolecules	LBG, NHLBI
OTHERS:	Tadashi Yanagisawa	Visiting Fellow	LBG, NHLBI
	Fiorenzo Battaini	Visiting Fellow	LBG, NHLBI

COOPERATING UNITS (if any)

None.

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Laboratory of Biochemical Genetics

SECTION
Section on Macromolecules

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	2.5	0

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Studies of the biology of thyrotropin releasing hormone have continued. The metabolite of thyrotropin releasing hormone called histidyl-proline diketopiperazine has been the focus of our attention. We have accumulated further evidence that this compound is biologically active. This year we showed that histidyl-proline diketopiperazine is a non-competitive inhibitor of dopamine uptake into synaptosomes. An exploration of the mechanism of the inhibition of catecholamine transport by the peptide revealed that histidyl-proline diketopiperazine inhibits the sodium-potassium dependent ATPase that generates the ion-gradient necessary for catecholamine uptake.

PHS-6040
(Rev. 10-76)

Project Description: Z01 HL 00152-06 LBG

Major Findings:

Our efforts to explore the biological activity of histidyl-proline diketopiperazine have continued. This peptide was discovered by us approximately five years ago as a metabolite of thyrotropin releasing hormone. Our previous studies showed that the peptide is more active than thyrotropin releasing hormone in antagonizing the sleep-inducing effects of ethanol in rats, suggesting that the diketopiperazine might be a natural antidepressant. We have also shown that histidyl-proline diketopiperazine lowers the body temperature of rats that have been exposed to the cold and that thyrotropin releasing hormone antagonizes the hypothermic effect of the diketopiperazine. We have further shown that both thyrotropin releasing hormone and histidyl-proline diketopiperazine, when injected intraperitoneally into rats, leads to an elevation of cyclic GMP levels in the brain.

Since the biochemical basis of central nervous system depression, thermoregulation and regulation of cyclic nucleotide levels has at times been associated with catecholamine metabolism, the possibility that the effects of thyrotropin releasing hormone or histidyl-proline diketopiperazine on these processes might be mediated by effects on catecholamine biochemistry was explored. Tests of dopamine uptake in striatal synaptosomes from rats revealed that thyrotropin releasing hormone did not affect this process, but histidyl-proline diketopiperazine produced an inhibition. A study of the kinetics of the inhibition showed it to be noncompetitive. Since the transport of dopamine is sodium-dependent, the possibility that the inhibitory effect of the peptide was at the level of sodium-potassium ATPase was examined. We showed that concentrations of histidyl-proline diketopiperazine that inhibited dopamine uptake also inhibited sodium-potassium ATPase. Exploration of the mechanism by which the peptide inhibites the ATPase enzyme indicated that the portion of the enzyme that communicates with the external environment that is inhibited by ouabain is not sensitive to the peptide. However, the portion of the ATPase that interacts with the cell interior that is inhibited by vanadate is modified by histidyl-proline diketopiperazine. The sensitivity of sodium-potassium ATPase to inhibition by histidyl-proline diketopiperazine may provide a basis for understanding the abundance of effects of this peptide.

Proposed Course of Research:

Our observation that histidyl-proline diketopiperazine inhibits sodium-potassium ATPase suggests that this peptide may be a general regulator of cell membrane properties. We look forward to exploring some other ways to check membrane effects of this peptide. We also hope to develop some methods to trace the possible changes in cellular concentration of histidyl-proline diketopiperazine under a variety of physiological conditions.

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Publications:

1) Battaini, F. and Peterkofsky, A.: Histidyl-proline diketopiperazine, an endogenous brain peptide that inhibits (Na^+ & K^+)-ATPase. Biochem. Biophys. Res. Commun. 94: 240-247, (1980.)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do **NOT** use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE **NOTICE OF INTRAMURAL RESEARCH PROJECT**	PROJECT NUMBER Z01 HL 00153-02 LBG

PERIOD COVERED
October 1, 1979 - September 30, 1980

TITLE OF PROJECT (80 characters or less)

Biochemical characterization of α-adrenergic receptors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Steven L. Sabol	Medical Officer	LBG NHLBI
OTHERS:	Daphna Atlas	Visiting Scientist	LBC NIAMDD

COOPERATING UNITS (if any)

Laboratory of Bio-organic Chemistry, NIAMDD

LAB/BRANCH
Laboratory of Biochemical Genetics

SECTION
Section on Molecular Biology

INSTITUTE AND LOCATION
NHLBI, NIH, Bethesda, Maryland 20205

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☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

A program was begun to devise novel molecular probes and affinity labels for α-adrenergic receptors. Several analogues of clonidine (an α_2-adrenergic receptor partial agonist), and other α-adrenergic ligands were synthesized and tested for binding to α-receptors of rat brain and NG108-15 neuroblastoma x glioma hybrid cells. In the latter system, α-receptor binding of [^{3}H]clonidine was partially characterized and correlated with α_2-receptor-mediated inhibition of adenylate cyclase. A hydroxyphenacetyl derivative of p-aminoclonidine was prepared as an α-receptor ligand that may be iodinated; this compound possesses both strong α-receptor partial agonist activity and moderate opiate-receptor agonist activity.

PHS-6040
(Rev. 10-76)

Project Description:

Objective:

Little is known about the molecular properties of α-receptors. Recent work has resulted in the differentiation of at least two classes of α-receptors, α_1 and α_2. Clonal NG108-15 neuroblastoma x glioma hybrid cells possess α_2 receptors that mediate the inhibition of adenylate cyclase. We previously proposed that activation of α_2-receptors, but not necessarily α_1-receptors, may be generally associated with the inhibition of adenylate cyclase. One approach to the biochemical characterization of α-receptors is to prepare suitable affinity labels that will specifically react covalently with either α_1 or α_2-receptors. In addition, there is a need for radioiodinated and fluorescent α-receptor probes.

Major Findings:

Clonidine, p-aminoclonidine, and N-(4-hydroxyphenacetyl)-4-aminoclonidine (HP-aminoclonidine) were shown to be potent partial agonists in the α-adrenergic-receptor mediated inhibition on NG108-15 adenylate cyclase (K_{Dapp} 300, 50, 130 nM, respectively); these compounds also bind with high affinity to α_2-receptors of rat brain and NG108-15 membranes, as determined by displacement of bound [^{3}H]clonidine. For brain membranes, binding of [^{3}H]-clonidine that is displaceable by the α-antagonist phentolamine has been previously characterized as binding to α_2-receptors. We characterized [^{3}H]-clonidine binding to NG108-15 membranes in order to correlate binding affinities with inhibition of adenylate cyclase in the same system. [^{3}H]Clonidine binds to NG108-15 membranes with K_D values of 1.7 and 33 nM for apparent high- and low affinity sites, respectively. The relative proportions of these apparent classes of sites varied among different preparations. Classical α-adrenergic agonists and antagonists displace [^{3}H]clonidine from these sites. p-Aminoclonidine and HP-aminoclonidine displace [^{3}H]clonidine from the high-affinity sites with K_D values of 2.3 and 5.8 nM, respectively. Similar affinities of the analogues for rat brain α_2-receptors were found. Radioiodination of HP-aminoclonidine was attempted, but so far without retention of α-adrenergic activity.

In the NG108-15 system, the affinities of ligands for the high-affinity α-receptor binding sites were found to be many times greater than the corresponding potencies in the inhibition of NG108-15 adenylate cyclase. This discrepancy can be explained by the findings that GTP, ATP, Na^+ ions, and possibly other components of the adenylate cyclase system inhibit binding of α-receptor agonists to the high affinity site. GTP and probably Na^+ ions are required for α-receptor-mediated inhibition of adenylate cyclase; thus, the high-affinity [^{3}H]clonidine site may represent uncoupled receptors, while the low affinity sites may represent receptors coupled to adenylate cyclase.

HP-aminoclonidine possesses opiate receptor agonist activity in addition to α-receptor partial agonist activity. This analogue inhibits NG108-15 adenylate cyclase (K_D 0.7 μM), and most of the inhibition is reversed by opiate

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receptor antagonists. HP-aminoclonidine also displaces [^{3}H]dihydromorphine and [^{3}H]D-Ala-Met-enkephalinamide from rat brain opiate receptors with IC_{50} values of 0.6 and 0.12 μM, respectively. In one preliminary experiment, HP-aminoclonidine, when injected subcutaneously in mice, elicited analgesia; the receptor mediating this effect has not been fully characterized.

In addition, other compounds were synthesized as potential affinity labels for α_2-receptors. These included bromo-acetyl and fluorosulfonyl derivatives of amino-clondine, yohimbine, and phentolamine. Several promising compounds are currently under evaluation by Dr. Atlas, now back in Israel.

Significance to Biomedical Research:

Alpha-adrenergic receptors mediate responses to norepinephrine and epinephrine in the autonomic nervous system and in a variety of non-neuronal cells. The use of affinity labels, particularly those preferring α_1 or α_2-receptors, should enable the identification of the molecules constituting the receptors. The potent α-receptor activity of the analogue HP-aminoclonidine is an indication that the amino group of p-amino-clonidine will accept modification by potentially reactive or fluorescent groups with retention of biological activity. This will be useful to produce a variety of α-receptor probes of high affinity.

The NG108-15 system is one of the best available systems to study α_2-receptor-adenylate cyclase interaction. It may serve as a model to study presynaptic α-receptors that regulate norepinephrine release in the sympathetic nervous system. These receptors may be involved in blood pressure regulation and in the etiology or pathogenesis of essential hypertension.

The opiate-receptor activity of HP-aminoclonidine is interesting because the compound does not closely resemble other opioid compounds, although its structure possesses several elements thought to be required for opiate activity.

Proposed Course:

The development of useful, affinity labels for α-adrenergic receptors is being pursued by Dr. Atlas, who has returned to the Hebrew University, Jerusalem, Israel. Suitable compounds should be tested in the NG108-15 system, and this may necessitate future collaboration with the NIH.

Publications:

1. Atlas, D. and Sabol, S.L.: A novel analogue of clonidine with opiate-receptor agonist activity, Biochem. Biophys. Res. Comm. 94, 924 (1980).

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*U.S. GOVERNMENT PRINTING OFFICE : 1980 0-311-301/3181

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